Developmental and Genetic Factors Affecting the Efficacy of Codeine.

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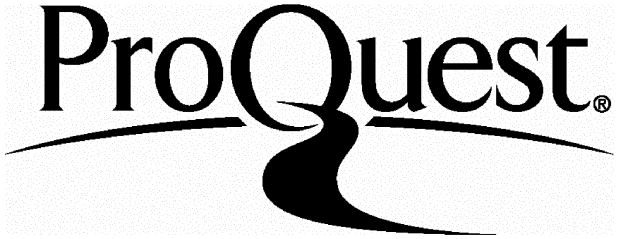

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Abstract

Codeine is widely used for analgesia in paediatric medicine due to the perceived benefits of good pain relief combined with a low incidence of opioid related side-effects. Experimental evidence indicates that the analgesia obtained from codeine is either entirely or almost entirely dependent on its metabolism to morphine.

This reaction is catalysed by the cytochrome P450 enzyme CYP2D6, which shows genetic polymorphism and decreased activity during early development. The aim of this thesis was to investigate how development and genetic variation may affect the ability of CYP2D6 to convert codeine to morphine and hence the efficacy of codeine.

A rat model was used to look at the effect of development on efficacy. Rats at different stages of development were treated with either codeine or morphine and were subjected to one of a range of noxious stimuli. Neonatal rats, in whom the enzyme activity is low, showed a markedly reduced response to codeine compared to older animals treated with codeine and animals of all ages treated with morphine. However, analysis of plasma for morphine levels from these neonatal animals following codeine was inconclusive and no relationship between plasma morphine levels and effect could be found at any age.

The effect of genetic variation on efficacy was investigated in a clinical study using children undergoing adenotonsillectomy. Children received either codeine or morphine and the analgesic effect was compared between the two groups, using two pain assessment tools, and also with plasma morphine levels and the genotype of the patient. Overall morphine and codeine appeared to provide equivalent analgesia but codeine showed more variability in its effect and there was a subgroup of patients in whom it appeared to provide little analgesia. However, it was not possible to link reduced efficacy to low plasma morphine levels or genetic variation.

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Abbreviations

AMPA	Amino-3-hydroxy-5-methyl-4-isoxazolepropriomic acid
ANOVA	Analysis of variance
ASA	American Society of Anesthesiologists
ATP	Adenosine tri-phosphate
AUC	Area under the curve
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
CGRP	Calcitonin gene related peptide
CHEOPS	Childrens Hospital of Eastern Ontario pain scale
CI	Confidence interval
CNS	Central nervous system
CSF	Cerebrospinal fluid
DA	Dark Agouti
DNA	Deoxyribonucleic acid
E	Embryoinic day
EAAs	Excitatory amino acids
ECF	Extracellular fluid
EM	Extensive metaboliser
EPSCs	Excitatory post synaptic currents
G	Gauge
GABA	γ-amino-butyric acid
GI	Gastrointestinal
HPLC	High performance liquid chromatography
HT	High threshold
IL	Interleukin
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
IM	Intermediate metaboliser
LT	Low threshold

M	Morphine
mRNA	Messenger ribonucleic acid
M-3-G	Morphine-3-glucuronide
M-6-G	Morphine-6-glucuronide
n	Number of subjects
NADPH	Nicotinamide adenine dinucleotide phosphate
ND	Not detected
NGF	Nerve growth factor
NK	Neurokinin
NMDA	N-methyl-D-aspartate
NNT	Number needed to treat
NO	Nitric oxide
NR	No result
NSAIDs	Non steroidal anti-inflammatory drugs
NT3	Neurotrophin 3
P	Postnatal day
PCR	Polymerase chain reaction
PG	Prostaglandin
PM	Poor metaboliser
r	Correlation coefficient
SD	Sprague Dawley
SG	Substantia gelatinosa
SP	Substance P
SSRIs	Selective serotonin reuptake inhibitors
TNF	Tumour necrosis factor
UDPGTs	Uridine diphosphate glucuronosyltransferases
vFh	von Frey hair
VIP	Vasoactive intestinal peptide
WDR	Wide dynamic range
5HT	5-hydroxytriptamine

CHAPTER 1. INTRODUCTION

1.1 Pain

The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage".(Merskey H 1979) It is thus a subjective experience that assumes both consciousness and the capacity for expression and action.

Pain functions as a protective mechanism to avoid potential tissue damage and to prevent damaged tissue from receiving additional injury, thus promoting the healing process. However, pain is not a uniform experience, and both the quality of pain and the initiation of protective responses are determined by many factors within the spinal cord and in higher centres involved in the integration and modification of nociceptive signals.

1.1.1 Pain in children

As with adults children can experience pain in many situations, including during illness or medical treatment, and should be given appropriate analgesia to attempt to relieve their pain. The Human Rights Act (1998) states that a person should be protected from inhumane or degrading treatment. As clinicians we thus have an obligation to our patients, be they adults or children, to use our knowledge and available resources to attempt to relieve their suffering, of which pain can be a major component.

Although it may seem obvious to the modern day clinician, the belief that even the most immature children can experience pain has only recently gained credence. This has lead to the under-treatment of pain and unnecessary suffering in this age group. Two reports in the 1980`s showed that children were often prescribed and given much less analgesia than their older counterparts, especially after surgery.(Schechter NL et al 1986; Mather L et al 1983) The reasons for this have included misconceptions about the nature of pain in infants and children, fear of inducing serious side effects and a lack of acceptable alternatives to painful intra-muscular injections. The findings from these

studies have promoted extensive interest in the experience and effects of pain in children and have shown the extent of the mismanagement of pain in this group. There is now a large body of literature on the subject of paediatric pain and its management and attitudes of clinicians have changed. However, much is still unknown about the genesis and treatment of pain in children, especially in terms of development, and more work to further our understanding is required.

Many common analgesic drugs and regimens have not been sufficiently investigated in infants and children. There are relatively few randomised controlled trials of treatment, and management is often empirical with no reference to systematic investigation. Children are not a defined population and differences between pre-term neonates, neonates, infants, pre-verbal children, pre-school children, older children and adolescents mean potentially different requirements for acceptable pain management strategies.

Advances in developmental anatomy and neurobiology have been responsible for major changes in the approach to paediatric pain management and research. It is now well established that infants are not simply scaled down adults and present a special case in themselves. We are also now aware that early development is not simply a state of immaturity, in the sense of "a lack of or incomplete function", but that the developing organism is fully functional and extremely well adapted to its primary role i.e. the process of growth and development.

The implication of this second premise is that many neurobiological systems such as neuronal fibre tracts and neurotransmitter and trophic signalling systems are in a more dynamic, "plastic", state than in the adult. This state of extended function and evolving anatomy is present in all systems involved in the processing of noxious stimuli e.g. the central and peripheral nervous systems, the immune system and skin. Examples of this dynamic state are seen at all points on the pain pathways from peripheral nociceptors through to higher brain centres. More work is required to understand how this plasticity affects and is affected by tissue injury, the perception of pain and the effects of analgesia.

Although there is a growing body of literature into paediatric pain and its management there are still constraints not present in adult practice. This is true not only for pain and its treatment but for paediatric medicine in general and is a reflection of the methodological, ethical and economic difficulties involved in research in children.(Conroy S et al 2000; Conroy S et al 1999)

Pain assessment. The vast range of physiological and behavioural responses, cognitive abilities and psychological development between the pre-term neonate and mature adolescent poses enormous problems for valid and reliable pain measurement. There is extensive literature on paediatric pain assessment and in the neonate and infant alone 14 different pain assessment tools have been devised and validated.(Buttner W et al 2000; Franck LS et al 2000) There is also little consensus on which tool is superior for different groups or situations.(Clinical Guidelines for the Recognition and Assessment of Acute Pain in Children. 1999)

Pain in adults is usually defined in terms of subjective "self" report which is generally specific but not necessarily very sensitive. However the need for understanding and cognitive development make this type of report unsuitable for younger children, especially preverbal children such as neonates and infants. In these children pain related behaviours are looked for and a variety of tools have been developed using behavioural, physiological and metabolic variables. However, these may also be affected by maturity or modified by other factors such as disease state, mood, anxiety, distress, somnolence and concurrent drug therapy. In addition observer related factors may also be important; health care workers and parents have been shown generally to underestimate children`s pain in comparison with self-report methods.(Romsing J et al 1996; Jyli L et al 1995; LaMontagne LL et al 1991)

Thus the measurement of pain in children is difficult, being neither necessarily specific or sensitive, and the information obtained must always be examined and interpreted with these limitations in mind.

Pain perception. Studies must necessarily make assumptions or indirect conclusions regarding pain perception and memory that cannot be directly tested. Due to our lack of

knowledge it is very difficult and complex to make assumptions or to try and define suffering in neonates and infants.

Economics. At present there is little economic imperative to treat paediatric pain. Thus the drive for research into this area is not great. However, further information on the long term consequences of treating or not treating pain may lead to more powerful economic arguments for paediatric pain research and management.

Differences in pharmacology. Pain management interventions in neonates and infants may be limited by the perceived potential for adverse reactions and side effects, whether there is conclusive evidence for them or not. With so little known about the pharmacology of some drugs when used in this age group there maybe ethical problems enrolling children into trials of these therapies and thus a delay in the time taken to gather information regarding efficacy, safety and long-term consequences.

At present treatment of pain in children is very much based on those techniques used in adult practice. It is thought that multi-modal or balanced analgesia using both pharmacological and psychological methods, which act on different components of the pathways of the response to pain and injury, is likely to be the most effective.(Kehlet H et al 1999; Kehlet H 1997) However, in children there is still a lack of information regarding appropriate dosages and potential toxicity of many drugs. Balanced analgesia encourages the addition of new agents in order to improve pain relief rather than increasing the dose of single drugs thereby exploiting synergistic effects and minimising unwanted effects. Despite the lack of systematic evaluation of balanced analgesia in children it remains the most popular approach on both empirical and theoretical grounds.

As clinicians treating pain in children we have to try and balance our duty to the care of the patient with our lack of theoretical knowledge and evidence based information in many areas. The difficulties in assessing and measuring pain in younger children means that we must be as confident as possible of the efficacy and safety of the analgesic interventions we prescribe to avoid unnecessary suffering. Thus we must continue to investigate the effect of development in all areas of pain and not assume that adult practices can be simply "scaled down" for use in children.

In the rest of this chapter I will present an overview of our current knowledge on pain processing and pathways and opioid systems and what is presently known about their development.

1.1.2 Peripheral pain perception(Fitzgerald M et al 2001; Abram SE 1998; Dray A 1997)

Peripheral tissues contain receptors, called nociceptors, that are either excited by or respond preferentially to noxious stimuli. These specialised receptors transduce physical energy of high intensity into action potentials that travel to the central nervous system via the peripheral nervous system, along primary afferent nerve fibres and provide information on the location, quality and intensity of stimulation. They are located on free nerve endings and are not encapsulated.

The cell body of a primary afferent neuron is located in the dorsal root ganglion or in certain cranial nerve ganglia. A peripheral process travels to a target in peripheral tissue via a peripheral or cranial nerve. A central process terminates in the spinal cord. There are two general classes of nociceptive afferent nerve fibres based on conduction velocities. The Aδ fibres, which are thinly myelinated and conduct at rates between 2 and 30 metres per second, and C fibres, which are unmyelinated and conduct with a velocity of less than 2 metres per second. The nociceptors of both Aδ and C fibres are further sub grouped functionally according to the natural noxious stimuli that excite them i.e. temperature, mechanical and chemical stimuli. Some receptors may respond to both temperature and mechanical stimuli. These receptors have been found in visceral tissue and skeletal muscle as well as cutaneous tissue. In addition mechanical skin stimulation may also activate low threshold Aβ fibres.

Most nociceptors encode the intensity of noxious stimuli. The greater the intensity of stimulation, the greater the discharge.(Garell PC et al 1996; LaMotte RH et al 1978) The quality of pain sensation depends on the type of nociceptor. Excitation of cutaneous C fibres typically evokes a sensation of burning or dull pain whereas Aδ nociceptors are typically associated with sharp pricking pain.

Inflammation(Fitzgerald M et al 2001; Abram SE 1998; Dray A 1997)

When significant tissue damage occurs, such as following surgery or trauma, pain is often more persistent and is associated with inflammation. Inflammation is characterised by an altered perception of pain (hypersensitivity) itself characterised by enhanced pain sensation to a noxious stimulus (hyperalgesia) and an abnormal sensation of pain to a previously non-noxious stimuli (allodynia). The pain may also be spontaneous and ongoing. Hyperalgesia following tissue injury can also be sub divided into primary hyperalgesia, which develops at the site of injury and appears to arise largely from peripheral nociceptor sensitisation, and surrounding secondary hyperalgesia, which is thought to arise from central plastic changes in spinal cord connectivity modifying central nervous system (CNS) responsiveness to future stimuli.

Inflammation involves a complex series of biochemical events following tissue damage, which contribute to the healing process. During an inflammatory response many substances are released into the injured area from a variety of sources, including damaged cells, immune cells, circulating leukocytes and platelets, vascular endothelial cells and the peripheral and sympathetic nervous system.(Levine J et al 1994; Handwerker HO 1976) They act via a multiplicity of receptors coupled for the most part, with a limited repertoire of cellular regulatory intermediates i.e. G proteins and secondary messengers, to regulate permeability and cellular ion concentration. This results in the classical signs of inflammation: redness, heat, swelling and pallor.

These inflammatory mediators can be characterised according to where they are generated:

1. Mediators generated by tissue damage and inflammation. These include hydrogen and potassium ions, nitric oxide (NO), nerve growth factor (NGF), adenosine, adenosine tri-phosphate (ATP), kinins (bradykinin and kallidin), prostinoids (prostaglandin(PG)E_2 and PGI_2), 5-hydroxytryptamine (5-HT) and histamine.

2. Mediators released from neurones. The nuerokinins (substance P and neurokinin A), neuropeptide Y, noradrenaline and calcitonin gene related peptide (CGRP) are released from nerve endings during inflammation.

3. Mediators released from immune cells. The cytokines interleukin(IL)-1, IL-6, IL-8 and tumour necrosis factor(TNF)α are released from immune cells and induce hyperalgesia indirectly by release of prostinoids from several tissues, increasing the expression of NGF or kinin receptors and affecting the neurochemistry of sympathetic fibres.(Abram SE 1998; Dray A 1997)

The mediators may directly activate peripheral nociceptors to cause pain but more often they act indirectly to sensitise nociceptors and alter their response properties to subsequent stimuli.(Yaksh TL 1999; Woolf CJ et al 1999a) This occurs by modification of receptor molecules or voltage gated sodium channels by transcriptional and posttranslational events to reduce the activation threshold and thus allow lower intensity stimuli to evoke pain and a greater response to be evoked. These changes in gene transcription may induce changes that are long-term and are thus a factor in the production of ongoing pain. Another explanation maybe that formerly "silent" nociceptors are recruited by the release of these active mediators.

C fibres also play a part in the genesis of inflammation by an efferent function. When a C fibre is excited action potentials are not only transmitted to the CNS, they also travel antidromically along the peripheral branches of the nerve endings and cause the release of neuropeptides from the nociceptor terminal. The vasoactive neuropeptides e.g. substance P, produce inflammation via vasodilation and plasma extravasation, called neurogenic inflammation. Neuropeptides released from one nociceptive ending can excite adjacent endings of other nociceptive fibres in a process known as the axon reflex.(Abram SE 1998)

Development of nociceptors

Very little is known about the developmental regulation of the nociceptors and nociceptive signal transduction.(Fitzgerald M et al 2001; Alvares D et al 1999) More is

known about general anatomical and physiological development though the majority of the work has been done using animal models.

Sensory nerve fibres terminating in nociceptive or low threshold mechanoreceptor endings grow out from the dorsal root ganglion prenatally and innervate the skin in an organised proximo-distal manner. By birth in the rat and the second trimester in man, they have reached the most distal skin of the foot.(Payne J et al 1991; Reynolds ML et al 1991) The large diameter A cells are born first and their nerve fibres reach the skin and form the initial cutaneous nerve plexus before the C fibres which follow soon after.(Jackman A et al 2000) Two groups of C nociceptors have been distinguished. The first contain neuropeptides and express the neurotrophin receptor for NGF, trkA. The second do not but bind the lectin IB4 and express the receptors for the neurotrophin GDNF. The functional differences between these two groups is not known but in the rat the IB4+ group only mature postnatally, so that in early life the majority of C nociceptors express trkA.(Bennett DL et al 1996)

All the main functional cutaneous afferent types found in the adult rat hindlimb can be found at birth.(Fitzgerald M et al 1992; Fitzgerald M 1987a) C fibre polymodal nociceptors, responding to mechanical, thermal and chemical noxious stimuli, are fully mature in their thresholds, pattern and frequency of firing at birth. Aδ mechanoreceptors, responding maximally to noxious mechanical rather than chemical and thermal stimulation, can also be distinguished, but their peak firing frequencies are lower than in adults. Low-threshold Aβ mechanoreceptors responding to touch or brush with brief, rapidly adjusting bursts of spikes are, relatively, the most immature at birth, with low frequencies of firing and amplitude of response.

Neurotrophins also play an important part in nociceptor development. The number of sensory afferents responding to a noxious stimulus, and their pattern and amplitude of response, will depend upon the level of neurotrophic factors in the skin or other target tissues.(McMahon SB 1996; Snider WD 1994) There is a continuous cycle of neurons being born and innervating the skin alongside programmed cell death and the survival of neurons is dependent upon access to neurotrophic factors.(Coggershall RE et al 1994; Ruit KG et al 1992) For example, C nociceptor neurons that express trkA depend on NGF for survival and gene deletion or mutation of trkA expression can lead to a rare

sensory and autonomic neuropathy with a congenital insensitivity to pain.(Indo Y et al 1996) In addition, neurotrophic factors also play an important role in the development of normal receptor properties independent of their effect upon survival. NGF and neurotrophin 3 (NT3) regulate the differentiation of myelinated and C fibre nociceptors and their mechanical thresholds and brain derived neurotrophic factor (BDNF) regulates mechanoreceptor properties.(Koltzenburg M 1999) Neurotrophin levels also determine the innervation density of the skin and access to excess NGF and BDNF leads to skin hyperinnervation.(LeMaster AM et al 1999; Albers KM et al 1994)

1.1.3 Central nociceptive pathways(Fitzgerald M et al 2001; Abram SE 1998)

A noxious stimulus is required to activate nociceptors but the production of a sensory response and ultimately perception will depend upon the ability to excite neurons within the central nervous system (CNS). The proliferation of research into adult pain mechanisms has demonstrated that pain transmission in the spinal cord is not through a simple circuit, but rather the arrival of noxious information from the periphery via primary afferent nerve fibres initiates a cascade of events in the dorsal horn allowing enhancement and modulation of the pain signal.(Dickenson AH 1995; Woolf CJ 1994)

Afferent fibres enter the spinal cord and travel in Lissauer`s tract before sending processes into the dorsal horn. Aδ fibres synapse in laminae 1 and 5, C fibres synapse in laminae 1 and 2 (the substantia gelatinosa (SG)) with some visceral C fibres synapsing in lamina 10 and in the contralateral deep dorsal horn. Aβ fibres synapse in laminae 3,4,5 and 6. They then activate projection neurons, either directly or via interneurons, which will decussate within a few segments. Descending fibres projecting from brainstem nuclei modulate the incoming information via interneuronal networks, thus making the dorsal horn a significant site for the processing of incoming noxious information.

Nociceptive information is then conveyed up the spinal cord in white matter tracts traditionally thought to be in the anterior funiculus. These tracts ascend to the sensory cerebral cortex via the medulla, midbrain and thalamus. En route projections are sent off to the medullary reticular formation, nucleus magnus raphe, midbrain reticular

formation, periaqueductal grey matter, ventrobasal complex and the medial and lateral nuclei of the thalamus.

Dorsal horn cells are divided into three simple types depending on their pattern of responses to mechanical stimuli:

1. Low threshold (LT) cells, which respond to a brush stimulus but not to a maintained mechanical pressure or a noxious stimulus, which receive input exclusively from low-threshold mechanoreceptors.

2. High Threshold (HT) cells, which respond to noxious stimuli only and receive input from C and Aδ fibres.

3. Wide dynamic range (WDR) cells, which respond over a range from innocuous to noxious in a frequency dependent fashion. They receive input from Aβ, Aδ and C fibres. This possibility of different afferent convergence on a single cell allows output frequency to be encoded by input intensity and thus parallel the perceived pain.

Pharmacolgy of central nociceptive transmission

The neurotransmitters involved in spinal nociceptive pathways have been the subject of intensive research.(Dickenson AH 1995; Levine JD et al 1993) The terminals of primary afferent fibres contain:

1. Peptides – substance P (SP), CGRP, somatostatin, cholecystokinin (CCK) and vasoactive intestinal peptide (VIP).

2. Excitatory amino acids (EAAs) – glutamate and aspartate.

They are contained in separate vesicles within the same primary afferent, though location of these vesicles appears to be distinct to the individual fibre. A noxious stimulus causes their release with subsequent binding to their respective postsynaptic receptor.(Yaksh TL et al 1994) EAAs produce a rapid early depolarisation whereas peptides tend to provoke a delayed and prolonged depolarisation. The pattern of

postsynaptic activity in the dorsal horn neurons will code the onset, duration, intensity and location of the stimulus.

SP has been shown to be involved in pain transmission and is released from primary afferents, interneurones and descending fibres.(Dickenson AH 1995) It is a member of the neurokinin family of neurotransmitters and is found in abundance in the dorsal horn. Three types of neurokinin receptor have been identified – neurokinin-1 (NK1), NK2 and NK3. They are located postsynaptically in laminae 1, 2 and10 of the dorsal horn and SP is thought to act preferentially on NK1.

Fast excitatory synaptic transmission in adult pain pathways is mediated by glutamate and aspartate acting on postsynaptic amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) receptors and kainate ligand gated ion channels.(Li P et al 1999)

Development of the spinal cord

Spinal cord neurogenesis takes place in a ventro-dorsal direction beginning with motorneurons and ending with the local interneurons in the superficial dorsal horn or SG.(Altman J et al 1984; Bicknell HRJ et al 1984) In the rat, both A and C fibres have grown into the spinal cord by birth but C fibre terminals are immature, shown by the fact that many C fibre specific markers are not apparent in the spinal cord until the prenatal period.(Jackman A et al 2000) Synaptogenesis in the rat dorsal horn is at its maximum in the first postnatal weeks whereas in primates all types of primary afferent terminal and postsynaptic specialisation occurs early in the embryonic period.(Knyihar-Csillik E et al 1999)

The growth of A and C fibres into the rat cord is somatotopically precise whereas laminar organisation is not.(Fitzgerald M 1985; Fitzgerald M et al 1983) In the adult rat Aβ afferents are restricted to laminae 3 and 4 but in the fetus and neonate their terminals extend dorsally to laminae 1 and 2. Gradual withdrawal from the superficial laminae occurs over the first three postnatal weeks.(Fitzgerald M et al 1994) Conversley, C fibres grow specifically to laminae 1 and 2 and thus for a long period these laminae are occupied by both A and C fibre terminals.(Fitzgerald M 1987b) A

fibres form synaptic connections whilst in the SG and destruction of C fibres in the neonatal rat with capsaicin leaves A fibre terminals in the SG thus showing that C fibres play an important role in the withdrawal of A-fibres from laminae 1 and 2.(Tornsey C et al 2000a; Coggershall RE et al 1996; Shortland P et al 1994)

In the newborn rat the synaptic linkage between afferents and dorsal horn cells is weak.(Jennings E et al 1998; Fitzgerald M 1985) Despite this a noxious stimulus will produce intense afferent activation of the dorsal horn cells, which leads to a rapid, transient central response. In immature rats, excitatory postsynaptic currents (EPSCs) can be elicited by Aβ afferents in SG neurons whereas in adults this is only possible with Aδ and C fibre afferents.(Park JS et al 1999) Expression of c-fos in SG neurons can also be evoked by innocuous inputs and A fibre activation at this age, but is only seen with noxious and Aδ and C fibre inputs in adults.(Jennings E et al 1996) Also the receptive fields of the dorsal horn cells of the newborn are larger. They occupy a relatively larger body surface area that gradually diminishes over the first 2 postnatal weeks.(Fitzgerald M et al 1999; Fitzgerald M 1985)

These large receptive fields and dominant A fibre input will increase the chance of central cells being excited by peripheral sensory stimulation and act to increase the sensitivity of infant sensory reflexes. During the first postnatal week, cutaneous reflexes are enhanced in rats and humans and non-noxious low threshold stimuli are able to elicit a flexion withdrawal reflex.(Fitzgerald M 1995; Fitzgerald M et al 1988)This excitability reduces to adult levels by postnatal day (P) 20 to P30 in the rat and by 30 weeks gestation in the premature human neonate.(Fitzgerald M 1997)

The postnatal maturation of synaptic connections between afferent C fibres and SG cells takes place over a prolonged period. C fibre activation is unable to provoke spike activity in the rat spinal cord until the second postnatal week and is unlikely to be involved in the immediate reaction to a noxious stimulus in young infants.(Nakatsuka T et al 2000; Fitzgerald M et al 1999; Fitzgerald M 1988) C fibres are able to produce subthreshold activation of central neurons and thus may trigger more persistent responses.(Akagi H et al 1983)

Development of central neurotransmission

AMPA and kainate receptors are expressed early in the developing spinal cord, and are evident in the first trimester of human development.(Akesson E et al 2000) Expression in the neonatal rat shows a wider distribution than in the adult and decreases over the first 3 postnatal weeks.(Jakowec MW et al 1995a; Jakowec MW et al 1995b) The Glutamate R1, 2 and 4 subunits are more abundant in the neonatal compared with the adult cord and the ratios of subunits are different. Different combinations of subunits will affect desensitisation, ionic permability and current/voltage relationships.

SP and somatostatin appear in the fetal spinal cord before the advent of synaptic transmission, which suggests that they have a role in spinal cord development, although the exact role is unknown.(Fitzgerald M 1997) Levels of both these two neurotransmitters increase until the second postnatal week and NK1 receptors do not reach adult levels until the second week of life.(Kar S et al 1995)

1.1.4 Central Sensitisation(Fitzgerald M et al 2001; Abram SE 1998)

The secondary hyperalgesia and allodynia seen following tissue damage is the result of central synaptic rather than peripheral receptor alterations. Following inflammation, a state of hyperexcitability exists in sensory neurons in the dorsal horn which is termed central sensitisation.(Woolf CJ et al 1994) The simplest form of this sensitisation is known as "wind-up" in which activation of central cells by repetitive Aδ and C fibres leads to an increase in the ratio of outgoing action potentials to incoming action potentials with each successive stimulus. Thus normal inputs have an extended and exaggerated response.(Woolf CJ et al 1999b) The resultant prolonged depolarisation allows previously ineffective inputs to activate the neuron and previously effective inputs to be even more so.

This is an example of "plasticity" within the CNS and current evidence shows that the N-methyl-D-aspartate (NMDA) glutamate receptor is primarily involved.(Woolf CJ et al 1999b; Dickenson AH 1997; Dubner R et al 1992) AMPA and kainate are postsynaptic to primary afferents. Binding opens monovalent cation channels (Na^+ and

K^+) to give depolarisation of the postsynaptic membrane, an effect that is short lived. However, the NMDA receptor is located postsynaptically to interneurons and is only activated by the repeated activation of nociceptors associated with tissue injury. It thus requires previous membrane depolarisation by the activation of AMPA and kainate receptors, which remove Mg^{2+} ions that normally block the channel of the NMDA receptor at resting membrane potential. Activation leads to the influx of Ca^{2+} as well as Na^+ ions. The increased intracellular calcium leads to increased synthesis of NO and PGE_2 which act as secondary messengers and increase the sensitivity of primary afferent neurons and cause them to release more neurotransmitter in response to a fixed stimulus. NO and PGE_2 have also been postulated to diffuse out of cells and effect changes in neighbouring cells.

Other neurotransmitters also play a role in the activation of NMDA receptors. Neuropeptides such as SP (acting on NK1 receptors) and CGRP and growth factors such as BDNF (acting on the trkB receptors) released by C fibres may potentiate the release of glutamate and its actions on the NMDA receptor. G-protein coupled receptors such as NK1 and glutamate receptors and receptor tyrosine kinases, such as trkB receptors, may also enhance NMDA currents via activation of protein kinase C.(Woolf CJ et al 2000)

The rise in intracellular Ca^{2+} ions can also lead to longer-term changes by the synthesis of novel genes. For example, after inflammation, A fibre neurons which do not normally express neuropeptides begin to express SP and BDNF and this may contribute to the allodynic response.(Ji RR et al 1999; Neumann S et al 1996)

The effect of this enhanced neurotransmission and hyperexcitability includes the enlargement of receptive fields, increased spontaneous activity, greater discharges to mechanical, thermal and chemical stimuli and sometimes decreased thresholds.(Ren K et al 1996) All of this leads to increased neuronal activity transmitted to supraspinal sites and the onset of persistent pain.

Development of central sensitisation

Our current knowledge in this area has been derived from work using animal models, mainly the rat. Repetitive C fibre input, as seen in adults, is not seen in neonatal rats. C fibre evoked activity is not seen in the dorsal horn in the first postnatal week and repetitive peripheral stimulation at C fibre strength has no observable effect on dorsal horn cell spike responses. "Wind-up" can be seen from P10 onwards with C fibre stimulation at three times normal in a small proportion of cells.(Knyihar-Csillik E et al 1999; Fitzgerald M et al 1994; Pignatelli D et al 1989; Fitzgerald M 1987b; Fitzgerald M 1985; Bicknell HRJ et al 1984; Fitzgerald M et al 1983) The proportion of cells able to show this response increases by P21 but is still low.(Fitzgerald M et al 1999) The role of the immature C fibre terminals is still unknown.

On the other hand stimulation of peripheral receptive fields on the hindlimb at twice the A fibre threshold can produce considerable sensitisation of the dorsal horn cells in the neonate. It is particularly apparent in younger animals, being present in 33% of cells at P6, 6% at P10 and absent by P21.(Jennings E et al 1998)

The developmental regulation of the NMDA receptor function in relation to central sensitisation has not been directly studied. NMDA dependent C fibre evoked depolarisation of spinal cord cells and "wind-up" of cells to repeated C fibre stimulation have been demonstrated in the young (8-14 days) spinal cord "in vitro" but the effects on A fibre sensitisation are not known.(Sivilotti LG et al 1995) Preliminary results on the neonatal cord suggest that the ability of dorsal horn cells to display enlargement of receptive fields, increased spontaneous activity and enhanced responses to mechanical, thermal and electrical stimuli differs from that in the adult cord.(Tornsey C et al 2000b)

Neonatal dorsal horn contains synapses that have NMDA receptors but no AMPA receptors called "silent synapses" due to their inability to function at resting membrane potentials. However, there is evidence that they can affect neuron excitability without co-activation of AMPA receptors.(Bardoni R et al 2000) These synapses disappear with age as NMDA and AMPA receptors colocalise.(Baba H et al 2000; Petralia RS et al 1999)

The neonatal spinal cord has a higher concentration of NMDA receptors in grey matter than older animals.(Gonzalez DL et al 1993) The spread of receptors is even across all laminae up to P10-12. Then higher densities are found in the SG and at P30 distribution is the same as in the adult. The affinity of the receptors for NMDA decreases with age as does NMDA evoked calcium efflux.(Hori Y et al 1994) The subunit composition of the NMDA receptor channel complex undergoes significant rearrangement during spinal cord development which may affect function.(Watanabe M et al 1994; Audinat E et al 1994)

The immature SP/NK1 system will affect central sensitisation in the infant but the mechanism is not known. SP levels are low at birth and increase with age. Conversely NK1 receptor density is maximal in the first 2 postnatal weeks and decreases with age. Distribution of these receptors also changes with age.(Kar S et al 1995)

As in the adult, inflammation leads to the expression of new genes in the neonate, though the pattern is different. CGRP expression is switched on in both A neurons and IB4+ C neurons in the dorsal root ganglion for 7 days following carageenan inflammation of the hindpaw at birth and only returns to normal once the inflammation has subsided.(Beland B et al 2000) This may suggest that the non-peptidergic A and C fibres have the ability to express peptides under some circumstances in the postnatal period.

1.1.5 Modulation of pain pathways(Fitzgerald M et al 2001; Abram SE 1998)

It is well established that descending pathways originating in higher centres can modulate the output of spinal nociceptive neurons. These pathways can act directly on pre- or postsynaptically or act on interneurons. Interneurons can also have inhibitory as well as excitatory effects. These pathways are activated in the presence of persistent pain.(Dubner R et al 1999)

Descending pathways release neurotransmitters such as 5-HT, noradrenaline and endogenous opioids to exert inhibitory control. At spinal level there are several classes

of local interneuron that contain one or more peptides such as enkephalins or inhibitory amino acids such as γ-amino-butyric acid (GABA) and glycine. Both these systems have pre- and postsynaptic actions on primary afferents and function by decreasing the release of primary afferent neurotransmitter or decreasing postsynaptic excitability of the second order neuron.

Additional descending pathways release excitatory amino acids (EAAs) and can either excite the inhibitory interneurons, leading to a decrease in pre- and postsynaptic activity, or excite the second order neuron, resulting in descending excitation. Inhibitory interneurons receive tonic excitation from Aβ fibres. Removal of inhibitory control leads to an increased discharge of the second order neuron following low-threshold input and may result in allodynia.

Development factors affecting modulation

Descending inhibitory controls are immature at birth.(Fitzgerald M 1991) Animals given a mid-thoracic transection before P15 are less affected that those transacted at older ages.(Weber ED et al 1977) Descending axons from brainstem projection neurons grow to the spinal cord early in fetal life but they do not extend collateral branches to the dorsal horn for some time and do not become functional until P10 in the rat.(Boucher T et al 1998; Fitzgerald M et al 1986) This may be due to low levels of neurotransmitters and/or the delayed maturation of interneurons. Maturation of descending inhibition may be dependent on afferent C fibre activity. Rats with altered C fibre activity have reduced inhibitory controls and high dorsal horn cell firing rates.(DeFilipe C et al 1998; Cervero F et al 1985)

GABA is an inhibitory neurotransmitter in the adult but in the neonatal dorsal horn it appears to have an excitatory effect and may function as a modulator of neuronal development rather than a neurotransmitter.(Fitzgerald M 1997; Leinekugel Y et al 1997; Malcangio M et al 1996) GABA expression alters during development peaking during the first two postnatal weeks and declining to adult levels by the third.(Schaffner AE et al 1993)

The lack of descending inhibition in the neonatal dorsal horn means that an important endogenous analgesic system that may "dampen" noxious inputs as they enter the CNS is lacking and thus their effects may be more profound than in the adult. Thus this may also contribute to the increased neuronal excitability and lower stimulus thresholds seen in the younger age groups. It also explains why stimulus produced analgesia from the periaqueductal grey matter is not effective until P21 in rats.(van Praag H et al 1991)

1.1.6 Higher brain centres

The activity of higher brain centres contributes to the experience of unpleasantness felt following a noxious stimulus. Sensory, autonomic, arousal and motor responses all contribute to the experience in both the meaning of the pain and in the context in which the pain presents.(Price DD 2000) Several ascending pathways and brain regions are thought to be involved but there is little consensus as to the extent of involvement of these areas in adults.(Davis KD et al 1998) Some pathways project directly from dorsal horn to the cortex via the brainstem and limbic system whilst others project to the cortex via multiple thalamic nuclei.(Price DD 2000)

Development of higher centres

The emotions involved in the understanding of a noxious stimulus and its relation to the present and future are obviously limited in the neonate and infant. There is also little known in terms of development and maturation of pathways to the higher centres and no information on the extent and degree of activation of these higher centres in neonates and infants.

In the rat, spinothalamic afferents reach the thalamus at embryonic day (E) 19 and thalamic axons also reach the cortical plate at E19. By P0 there is an organised plexus in the cortical plate and some axons have reached the marginal zone.(Erzurumlu RS et al 1990) At P2-5 non functional AMPA receptors are found ("silent synapses") which are converted to being functional at P8-9.(Isaac JT et al 1999) By P7 cortical cells are organised in columns but have larger receptive fields than in the adult which suggests a

lack of inhibition similar to that seen in the spinal cord.(Armstrong-James M 1975) The rat cortex remains immature for up to six weeks after birth.

The human cortex takes many years to develop fully and is still developing during adolescence. Thalamocortical axons are first seen at 22-34 weeks gestation but synaptogenesis continues for many years.(Mrzljak L et al 1988; Huttenlocher PR et al 1982)

How the development of higher centres and their connections affects the perception of pain and the pain process is unknown but is a vital part in the understanding of pain and may be of major clinical importance.

1.1.7 Opioid systems

Morphine, the active ingredient of opium, was first isolated in1806 by Sterturner who named it after Morpheus, the God of Dreams, but it was not until 1973 that the first opioid receptors were described.(Pert CB et al 1973; Terenius L 1973; Simon EJ et al 1973) Shortly afterwards endogenous opioid peptides were first identified.(Hughes I et al 1975) Since then our knowledge of the mechanisms of actions of opioids has greatly increased.(Kanjhan R 1995; Dickenson AH 1994; Pasternak GW 1993)

Opiods act by mimicking endogenous opioid peptides and binding with high affinity membrane receptors, which comprise three major groups: mu, kappa and delta, and each receptor can produce different effects. Sub-division of receptor groups has been proposed but remains controversial, although there is evidence of differential effects of delta receptors in mice.(Jiang Q et al 1991) Autoradiography has shown that each receptor has a distinct distribution in the brain.(Mansour A et al 1987) We have little knowledge of the specific function of individual receptors but evidence has accumulated that each one has a different role in pain modulation.(Dickenson AH 1991) Genetic techniques allow the role of each receptor in opioid function in vivo.(Iversen LL 1996) Mice lacking the mu opioid receptor do not show the normal responses to morphine and the delta and kappa receptors do not mediate the effects of morphine, thus suggesting that the effects of exogenous opioids are mediated via the mu

receptor.(Matthes HWD et al 1996) Mu and delta opioid receptor agonists have a positive antinociceptive interaction whreas mu and kappa opioid receptor agonists have an antagonistic interaction.(Ossipov MH et al 1997)

The endogenous ligands for opioid receptors are the opioid peptides, which can be divided into three groups, enkephalins, endorphins and dynorphins. All have some agonist activity at each of the receptors but each receptor possesses distinct ligand selectivity.(Kosterlitz HW 1985)

A number of mechanisms exist in the CNS that can mediate the effects of opioids, with the predominant effect of decreasing neuronal firing. Stimulation of neuron presynaptic opioid receptors reduces the release of excitatory neurotransmitters involved in the pain pathways.(Hori Y et al 1992) Stimulation of postsynaptic receptors inhibits neurotransmission by producing hyperpolarisation and thus reducing evoked activity.(Lombard M-C et al 1989) Opioids may also inhibit interneurones which were preventing activity in other inhibitory interneurones.(Dickenson AH 1994)

The spinal cord is an important site for opioid action. This is clinically evident in the profound analgesia produced by the subarachnoid and epidural administration of opioids. The majority of opioid receptors are located around C fibre terminals in the SG though lower numbers are found at deeper levels of the dorsal horn.(Dickenson AH 1994) In the rat, mu receptors predominate in the spinal cord, with delta and kappa receptors comprising less than one-third of the total and 50-70% of the total number of opioid receptors being located presynaptically.(Besse D et al 1990)

Opioid systems do not act in isolation. The peptide neurotransmitter CCK is found in spinal neurones and supraspinally and affects opioid function by acting as a "break" to endogenous opioid action. Application of CCK reduces the analgesic actions of morphine, which are enhanced by the use of CCK_B receptor antagonists.(Mao J et al 1995) CCK and NMDA receptors may also both be involved in the production of opioid tolerance. Animal studies have shown that production of hyperalgesia and opioid tolerance share common neurochemical pathways.(Mao J et al 1995)

Supraspinal opioid systems also contribute to analgesia by decreasing nociceptive input to the brain by altering ascending and descending control systems, therefore reducing the central perception of pain. Intraventricular and intracerebral injection of systemically inactive doses of opioids in humans provides evidence for the role of supraspinal sites in the production of analgesia and the localisation of supraspinal receptors.(Lazorthes Y et al 1988; Yaksh TL et al 1988) In rats, opioid receptors are widely distributed throughout the CNS, most densely in the brainstem, thalamus, amygdala, hippocampus and cortex.(Mansour A et al 1993) Mu receptors are found in the majority of these areas, whereas delta receptors are more restricted, found mostly in forebrain regions with poor binding in the midbrain and brainstem. Kappa receptors are widely distributed throughout the forebrain, midbrain and brainstem, comprising only 10% of the receptors in the rat brain, but up to one-third in humans.(Mansour A et al 1993) Opioid receptors have been shown to be situated at sites close to the origin of descending controls in the periaqueductal grey matter and the medulla and microinjection of opioids into these areas produces analgesia by their stimulation.

Opioids may also act peripherally and possess "local analgesic effects. In animals the peripheral analgesic effects of opioid antagonists have been shown in inflammatory pain models.(Stein C et al 1989) It is thought that inflammation initiates the axonal transport of opioid receptors to the periphery and increases their number on peripheral nerve terminals and inflammation disrupts the perineurium exposing neuronal opioid receptors.(Stein C 1995) Endogenous peptides are also produced by immune cells infiltrating the inflamed tissue.(Stein C 1993) Intra-articular morphine has been used with some success following knee surgery but the results are inconclusive.(Heine MF et al 1994; Dalsgaard J et al 1994)

Development of opioid systems

Opioid receptors and endogenous opioid peptides appear early in fetal life in the CNS of the rat but their development is non-uniform in the brain and spinal cord. In brain opioid receptors are present at birth in many areas. In the adult rat mu and delta receptors predominate but at P6 mu and kappa receptors are in the majority.(Leslie FM et al 1982) Delta receptors are low at birth and rise to a maximum by the third postnatal

week. A similar pattern is seen in the developing spinal cord.(Attali B et al 1990) Further work using autoradiography has shown mu receptors present in rat spinal cord at P0, increasing in density up to P7 and then gradually decreasing to P21. Kappa receptors closely followed the mu receptor pattern and both were diffusely spread throughout the cord. Delta receptors were first observed at P7 and continued to increase in density even after P21.(Rahman W et al 1998) Mu receptor binding sites are concentrated in the superficial laminae of the dorsal horn in adults but at P1 both superficial and deep laminae have high densities, which reaches a peak at P4 before decreasing to adult levels and distribution.(Rahman W et al 1998; Kar S et al 1995)

In animal models endogenous opioids appear in the brain before opioid receptors.(Ruis RA et al 1991; Pickel VM et al 1982) The different opioids develop at different rates with enkephalin levels increasing in the perinatal period. By P6-25 the concentrations of the opioids have risen to adult levels.(Bayon A et al 1979) In human fetal spinal cord enkephalin-like immunoreactive fibres have been detected as early as 10 weeks.(Charnay Y et al 1994)

Testing the analgesic efficacy of opioids in developing animals has proved difficult and produced differing results. Despite this analgesic efficacy has been demonstrated in developing animals. In some studies analgesic efficacy was much greater from morphine in the older pups (P14-21) than in the younger pups (P3-12) whilst another study showed increased efficacy at P3 decreasing up to P21.(Marsh DF 1997; Giordano J et al 1987; Barr G et al 1986) The reasons for this maybe due to concurrently altering nociceptive physiology, which means equivalent noxious stimuli at different ages may be difficult to achieve, difficulties in behavioural testing of young animals and the different behavioural tests employed.

Comparison of morphine, a mu agonist, with ketocyclazocine, a kappa agonist, in the developing rat showed that the mu agonist was more potent in producing analgesia to thermal stimuli as early as P3 increasing up to a maximum at P14. The kappa agonist was more potent at producing analgesia to a mechanical stimulus with an onset at P7-10,(Giordano J et al 1987) thus showing differential development of the opioid receptors. The use of morphine in neonatal rats with an inflammatory pain model

showed a similar analgesic efficacy at P3 as was shown in adults.(McLaughlin CR et al 1990)

The sedative effects of opioids may be a factor in the younger animals. However, a comparison of the analgesic effects of morphine with the sedative effects of a barbiturate in animals aged P1-20 with an inflammatory pain model showed analgesia from morphine which was quantitatively different from the sedation seen with the barbiturate.(Abbott FV et al 1995) Convulsive activity following opioids was also considered to be a problem in younger animals but a study using high dose morphine and looking at EEG and behaviour showed that although there were some electrographic spikes at P1,3 and 6 there were no behavioural convulsions, suggesting morphine is not as toxic as was thought.(van Praag H et al 1992)

Opioid systems play an important part in the regulation of neuronal development in the neonate. Neonatal rats given opioid antagonists showed increases in dendritic length compared with controls and the increase was greater with total receptor blockade. (Hauser KF et al 1989; Hauser KF et al 1987) Endogenous opioids can thus exert an influence on dendritic elaboration and spine formation. Further work suggests that endogenous opioids suppress astrocyte growth by a receptor mediated mechanism.(Steine-Martin A et al 1990)

Thus endogenous opioids act as inhibitory growth factors in neuronal development, probably by inhibition of DNA synthesis.(Vertes Z et al 1982) Morphine inhibits DNA synthesis in P1-4 rats, but not older rats, in vivo. However, in vitro these effects were not seen suggesting that the inhibitory effects of opioids are by an indirect mechanism.(Kornblum HI et al 1987)

Exposure to exogenous opioids may also have an effect on neuronal development. Rats exposed to repeated doses of morphine in the fetal (via the mother) and neonatal periods showed tolerance to the analgesic actions of morphine and a decrease in the density of brain mu opioid receptors but their was no significant change in delta and kappa receptor densities.(Tempel A et al 1988; Zadina JE et al 1986) In one study, however, by P14 the numbers were shown to be similar to controls.(Tempel A et al

1988) Prolonged treatment with morphine showed no further changes in receptor number than those seen in early neonatal life.(Tempel A 1991)

Mice lacking the mu opioid receptor gene had no analgesic effect from morphine but showed no morphological differences and had normal health and growth.(Matthes HWD et al 1996) This suggests that mu receptors alone are not vital for neuronal development and possibly other neurtransmitter systems can compensate in their absence to promote normal development.

Little is known about the effects of opioid administration in the neonatal period on the long term development of the CNS but owing to the continued and increasing use of opioids in this age group this is an area that requires more investigation.

1.2 Cytochrome P450 and CYP2D6

1.2.1 Drug metabolism(Baynes J et al 1999)

The majority of drugs are metabolised in the liver, though this can take place in other organ systems. Generally the metabolites produced are less pharmacologically active than the substrate drug. However, some drugs which may be inactive can be converted to their active forms as a result of metabolism. These are known as prodrugs, of which codeine is thought to be an example. Metabolising systems must be able to act on an infinite range of molecules due to the exposure of the human body to numerous drugs and environmental compounds. This is achieved by the enzymes involved having low substrate specificity.

Metabolism proceeds in two phases:

1. **Phase 1:** the polarity of the drug is increased by oxidation or hydroxylation catalysed by a family of microsomal cytochrome P450 oxidases.
2. **Phase 2:** cytoplasmic enzymes conjugate the functional groups from the first phase reactions, usually via glucoronidation or sulphonation.

1.2.2 Cytochrome P450(Zanger UM et al 2000; Baynes J et al 1999; Okita RT et al 1997)

The term cytochrome P450 refers to a family of heme proteins present in all mammalian cell types, except mature red blood cells and skeletal muscle cells. They catalyse the metabolism of a wide variety of structurally diverse compounds: endogenously synthesised compounds such as steroids and fatty acids, the cyotchromes for which are found in mitochondria, and exogenous compounds such as drugs, food additives and industrial by-products, these cytochromes being found in smooth endoplasmic reticulum. The cytochrome P450 system has far reaching effects:

1. Inactivation or activation of therapeutic agents
2. Conversion of chemicals to highly reactive molecules, which may produce unwanted cellular damage, cell death or mutations
3. Production of steroid hormones

4. Metabolism of fatty acids and their derivatives

Nomenclature

Designation of a particular protein as a cytochrome P450 depends on its spectral properties. This group of proteins has a unique absorbance spectrum, shown by binding carbon monoxide to the reduced heme protein. This produces an absorbance spectrum with a peak at approximately 450nm. Specific forms of cytochrome P450 differ in their maximum absorbance wavelength, with a range between 446nm and 452nm.

The many forms of cytochrome P450 are classified into various gene subfamilies according to their sequence similarities. Individual forms are given an Arabic number to designate a specific family, followed by a capital letter to identify its subfamily, followed by another Arabic number designating the individual P450 form e.g. 1A2 or 2D6. This is prefixed by the term CYP, which represents the first two letters of cytochrome and the first letter of P450, thus giving, for example, CYP1A2 or CYP2D6. Members of the same family share at least 40% amino acid sequence homology and members of the same subfamily share at least 55% sequence homology.

In man 12 cytochrome P450 gene families have been identified, of which 3 share the main responsibility for drug metabolism, designated CYP1, CYP2 and CYP3. Humans have approximately 60 unique P450 genes and to date 39 functional human genes have been sequenced, of which 19 are in families 1,2 and 3.(figure 1) Only half a dozen of these genes, including CYP2D6, are responsible for the metabolism of the majority of prescribed and "over the counter" drugs.(Nelson DR et al 1999) CYP3A constitutes the major proportion of cytochrome P450 expressed in the liver and is thus the family mostly responsible for the metabolism of drugs.

Overall reaction

The cyochrome P450 enzymes co-localise with the reduced form of the flavoprotein enzyme nicotinamide adenine dinucloetide phosphate (NADPH) cytochrome P450 reductase. The general reaction catalysed is:

$$NADPH + H^+ + O_2 + SH \longrightarrow NADP^+ + H_2O + SOH$$

Where the substrate "S" may be a steroid, fatty acid, drug or other chemical that contains an alkane, alkene, aromatic ring or hetrocyclic ring substituent that can serve as a site for oxygenation. The reaction is termed a monooxygenation reaction as only one of the two available oxygen atoms is incorporated into the substrate. In mammalian cells, cytochromes P450 serve as terminal electron acceptors in electron transport systems, which are present either in the endoplasmic reticulum or inner mitochondrial membrane. The cytochrome P450 proteins contain a single iron protoporphyrin IX prosthetic group, which binds oxygen, and the resulting heme protein contains binding sites for the substrate. For this to occur the heme iron must be reduced from its ferric (Fe^{3+}) to its ferrous (Fe^{2+}) form, which is catalysed by NADPH-cytochrome P450 reductase, so that oxygen can bind to the heme iron. A total of two electrons are required for this monooxygenation reaction. The electrons are transported to the cytochrome P450 individually, the first to allow oxygen binding and the second to cleave the oxygen molecule to generate the active oxygen species for insertion into the reaction site of the substrate.

Multiplicity of genes

Many cytochrome P450 forms have emerged due to gene duplication events occurring in the last 5 – 50 million years. The different forms of cytochrome P450 among various animal species are likely to have arisen from the selective pressure of environmental influences, such as dietary habits or exposure to environmental agents. Primordial genes have given rise to cyochromes P450 able to metabolise endogenous substances. Examination of the phylogenetic tree has shown that the earliest cytochromes P450 evolved to metabolise cholesterol and fatty acids.

Induction and Inhibition

The hepatic synthesis of cytochromes P450 can be increased by both exogenous and endogenous compounds. This process is known as induction and it increases the rate of phase 1 reactions. It has been shown to occur at both transcriptional and posttranscriptional level and it is not possible to predict the mode of induction based on the inducing compound. Enzyme induction can lead to altered drug efficacy of drugs via:

1. Increased rate of inactivation and/or enhancement of excretion.
2. Stimulation of the metabolism of the drug itself or other drugs that are substrates for the cytochrome P450 system
3. Unwanted side-effects due to the increased production of toxic metabolites

This can lead to potentially important clinical problems. For example, rifampicin will enhance the clearance of oral contraceptives due to its induction of CYP3A4. This leads to a decrease in effectiveness of the contraceptive drug and an increase in the incidence of unplanned pregnancy in women prescribed both drugs.

Conversely, drugs that form a relatively stable complex with a particular cytochrome P450 inhibit the metabolism of other drugs that are normally substrates for that cytochrome P450. An example of this is the inhibition of codeine metabolism by quinidine, though as discussed in Chapter 1, this inhibition provides a useful investigative tool when looking at the metabolism of codeine.

Polymorphisms

Individuals may also differ in their rates of metabolism of a particular drug due to differences in the cytochrome P450 genes they possess. Different forms of a cytochrome P450 may exist both between populations and within a population, which will alter the functional activity of the cytochromes P450 and thus the metabolic capability of the individual. These genetic polymorphisms can thus lead to a wide

spectrum of metabolic capabilities within a population, from those unable to metabolise the drug through to those who metabolise the drug relatively quickly, and thus to a variation in therapeutic efficacy within the population. These polymorphisms can occur via a number of different molecular mechanisms:

1. The normal gene and, hence, the normal enzyme and normal metabolic rate.
2. Gene deletions, gene conversions and single base mutations, which lead to the absence of the active enzyme and, hence, complete absence of metabolic capacity.
3. Amino acid substitutions that can give rise to;
    - An unstable enzyme and a low capacity to metabolise.
    - An altered active site, which can alter substrate specificity and thus lead to the possible formation of other metabolites.
4. Duplicated, multiduplicated or amplified genes leading to higher enzyme levels and increased metabolism.

1.2.3 CYP2D6 (Zanger UM et al 2000; Ctytochrome P450, subfamily IID; CYP2D 1999; Ingleman-Sundberg M et al 1999)

The CYP2D6 enzyme belongs to the family CYP2 and is the only functionally active isozyme of the CYP2D subfamily in humans. CYP2D6 accounts for, on average, up to 2 - 5% of the total hepatic cytochrome P450 content compared with CYP3A isoenzymes which account for up to 30%.(Shimada T et al 1994)However, its expression varies dramatically from person to person, ranging from undetectable in individuals classed as poor metabolisers (PMs) , very low in so-called intermediate metabolisers (IMs) to more than 100-fold higher levels in the most active extensive metabolisers (EMs).(Gonzalez FJ et al 1998; Zanger UM et al 1989; Zanger UM et al 1988)

CYP2D6 is usually considered to be a hepatic enzyme but CYP2D6 protein expression has been shown in several extrahepatic tissues including the GI tract and the lungs, though at lower levels.(Guidice JM et al 1997; Preuksaritanont T et al 1995) It has also been identified in rat and canine brain tissue and in human brain preparations (Allard P et al 1994; Tyndale RF et al 1991; Niznik HB et al 1990; Lee EJD et al 1989; Fonne-Pfister R et al 1987) Further evidence for CYP2D6 expression in the brain has been

obtained at the messenger ribonucleic acid (mRNA) level, but enzymatic activity has not been conclusively demonstrated.(Gilham DE et al 1997; Tyndale RF et al 1991)

Unlike the majority of the other P450 genes CYP2D6 is not inducible, although there is growing evidence associating the enzyme with a number of disease states. Enhanced activity has been associated with malignancies of the bladder, liver, pharynx, stomach and, particularly, cigarette-smoking related lung cancer. There has also been a link with chronic inflammatory diseases, such as rheumatoid arthritis and ankylosing spondylitis, and neurodegenerative disorders.(Marez D et al 1997; Caporaso N et al 1990; Law MR et al 1989; Caporaso N et al 1989; Roots I et al 1988) Enhanced CYP2D6 mediated metabolism of one or more dietary/environmental agents may form a reactive intermediate that plays a role in cancer initiation and /or promotion in various tissues.(Nebert DW 1997) Reduced enzyme activity is thought to be associated with an increased risk of Parkinson disease.(Parkinson Disease; PD 1999)

CYP2D6 polymorphism is responsible for variation in spartine/debrisoquine oxidation. This particular oxidative step is important in the metabolism of more than 30 drugs and environmental chemicals, including about 20% of commonly prescribed drugs e.g. tricyclic antidepressants, selective serotonin reuptake inhibitors (SSRIs), some neuroleptics, some antiarrhythmics and β-adrenoreceptor blockers as well as codeine and tramadol.(Brosen K 1993; Brosen K et al 1989)

The gene subfamily has been mapped to chromosome 22 at 22q13.1 and shows autosomal recessive inheritance. CYP2D6 activity ranges from complete deficiency to ultrafast metabolism, depending on which genetic variant is present.(Gough AC et al 1993) To date, 55 variants have been identified and classified.(Marez D et al 1997; Daly AK et al 1996) Most are rare with 87% of genotypes being accounted for by five different variants in the populations studied.(Sachse C et al 1998; Marez D et al 1997) Classification of individuals as PMs or EMs correlates with the genetic variant expressed and the presence of activity altering mutations in the gene.(Gaedigk A et al 1991; Heim M et al 1990; Kagimoto M et al 1990; Zanger UM et al 1988) This may be done by either phenotyping, using sparteine, debrisoquine or dextromethorphan as the probe drug, or by genotyping, using deoxyribonucleic acid (DNA) extracted from

leucocytes for a polymerase chain reaction (PCR) DNA test.(Schmid B et al 1985; Evans DA et al 1980; Eichelbaum M et al 1979)

Misclassification can occur in the presence of drugs which are inhibitors of CYP2D6 e.g. quinidine, metoclopramide, some neuroleptics, some SSRIs and some antiarrhythmics.(Brosen K 1993; Brosen K et al 1989) This is commonly due to competitive inhibition of the metabolism of compounds such codeine which have a relatively low affinity for the enzyme compared with these other compounds. This inhibition can result in the conversion of an individual from an EM to a PM.

Population genetics

The estimated population frequency of PMs in the UK is about 9%. However, there is wide variation among different ethnic groups, from about 1% in Arabs to 30% in Hong Kong Chinese.(Kalow W 1982; Evans DA et al 1980) Caucasians have an incidence of about 7%.(Alvan G et al 1990) Another study has confirmed this rate, with all the PMs being explained by 5 mutations.(Sachse C et al 1997) Further work using Caucasian PMs suggested only 4 alleles and gene duplication needed to be identified for reliable prediction of the PM phenotype.(Sachse C et al 1998)

There is a subgroup of EMs who are able to metabolise CYP2D6 substrates relatively quickly, known as ultra-rapid EMs. For drugs metabolised by CYP2D6 this may mean higher than normal doses are required for therapeutic effect or, in the case of a prodrug such as codeine, a potentially rapid onset and increased activity. (Bertilsson L et al 1993; Bertilsson L et al 1985) The frequency of ultra-rapid EMs also shows ethnic variation from 29% in Ethiopia to 1% in Swedish, German and Chinese populations.(Akullu E et al 1996; Yue QY et al 1995)

CYP2D6 and codeine – ethnicity

Studies comparing Chinese and Caucasian EMs have shown differences in the pharmacokinetics of codeine.(Bertilsson L et al 1992; Yue QY et al 1991; Yue QY et al

1989) The Chinese group were less able to metabolise codeine to morphine. Further work looking at codeine metabolism in three different Oriental populations found that the Chinese population metabolised codeine to morphine less effectively than Japanese or Korean populations, the metabolism in these latter two populations being very similar.(Yue QY et al 1995)

In addition variability can occur within ethnic groups. A study looking at the formation of morphine from codeine in Chinese subjects of 3 different genotypes showed subjects with different genotypes had identical serum codeine concentrations but that the morphine concentration in the urine varied between the genotypes. The clearance of morphine was between that of Caucasian EMs and PMs. The Chinese had an increased incidence of one enzyme mutation compared with Caucasians and, hence, a lower formation of morphine.(Tseng CY et al 1996) This indicates that different genotypes have different metabolic capacities for codeine by the O-demethylation pathway.

1.2.4 Development of the cytochrome P450 system(Oesterheld JR 1998)

Xenobiotic metabolising cytochromes P450 were first identified in fetal hepatic cell in the early 1970's.(Yaffe SJ et al 1970) Since then new research techniques (e.g. specfic antibodies, metabolic probes and inhibitors, polymerase chain reaction and recombinant cDNA enzymes) that are sensitive to very low levels of enzymes and express individual isoforms in quantity have allowed much more information on the development of the cytochrome P450 system to be gathered, though there is by no means a complete picture.

Fetal studies have concentrated on looking for either cytochrome P450 mRNA, that may predict functional cytochromes P450, or isolating and purifying cytochromes P450. This work has shown that these enzymes appear and become functionally active at different times ranging from the first trimester of the fetus through to infancy. CYP3A7 mRNA and CYP3A5 mRNA have been identified after only 42 days of fetal life and CYP1A1 mRNA has been detected as early as fetal day 45. These 3 enzymes have also been identified and purified as early as twenty weeks of fetal development. During the

second and third trimesters the mRNA of many more cytochromes P450 have been found.

The presence of fetal cytochromes P450 suggests that the fetus is an actively metabolising entity rather than the more historical view of the fetus as a passive entity protected by a maternal barrier. This ability of the fetus to metabolise can have both advantages and disadvantages. Molecules, such as ethanol and steroids can be detoxified, but the cytochromes P450 may also create metabolites that may be toxic to the fetus. These are often more water soluble than their parent compounds and can have difficulty crossing biological membranes and thus become trapped on the fetal side. This situation can be compounded by the fact the enzymes for phase 2 metabolism have not matured in the fetus. Such fetal reactions may be associated with carcinogenesis, mutanogenesis and dysmorphogenesis.

The expression of different cytochromes P450 changes over a lifetime. For example in the CYP3A subfamily high levels of CYP3A7 are present in the fetus but in the adult only small and variable amounts are expressed. Conversely, CYP3A4 only comes "on-line" after birth but in adults it is the predominant CYP3A. The amounts of different isoforms can also vary between individuals. Again looking at the CYP3A subfamily, CYP3A5 is more commonly expressed in children and adolescents than adults but it is not present in all children. These differences can have therapeutic consequences. Total hepatic activity of CYP3A of premature infants is one-third that of full term infants for up to 14 days after birth and thus the dosing requirements for midazolam (which is metabolised by CYP3A) is less for premature infants than term infants.(Burtin P et al 1994)

Total hepatic cytochrome P450 metabolism declines with age, though only particular cytochromes decrease and they go "off-line" at different times. There also seems to be a change in activity of some enzymes at the time of puberty e.g. prepubertal children require higher doses of psychotrophics than adults. It is thought that adult drug requirements are established at puberty, though the evidence is sketchy that this is due to changes in cytochrome P450 activity.

A good example of how development can affect the metabolism of particular drugs is caffeine. The metabolism of caffeine is complex and involves a number of different cytochrome P450 enzymes and metabolic pathways. The clearance of caffeine varies from fetal through to adult life depending on which of metabolic pathways is dominating, which in turn depends on the activity of the cyotchromes P450 involved. The activity of each enzyme develops at different times and can vary at different points during life thus giving rise to the metabolic differences seen.

1.2.5 Development of CYP2D6(Oesterheld JR 1998; Jacqz-Aigrain E et al 1992; Ladona MG et al 1991; Treluyer J-M et al 1991)

CYP2D6 mRNA has been shown to be present in fetal liver samples from the second trimester. The amount present gradually increases with fetal and neonatal age and reaches peak levels soon after birth. Levels remain high during the neonatal period and into infancy but decline during childhood to reach levels in adults similar to those found in fetal samples. This may suggest that CYP2D6 protein synthesis is less efficient in fetal and neonatal liver than it is in adult liver.

Most fetal liver samples have been shown to lack CYP2D6 protein, though it may be present in 30% of mid-trimester fetuses at much lower levels than after birth, approximately 5% of average adult levels. Postnatally there is an increase in the levels of CYP2D6 protein which is independent of gestational age (between 25 and 35 weeks) and appears to be directly related to birth. This is supported by the fact that only a small proportion of fetal samples contain the protein whereas all the samples taken after birth show evidence of the protein (except those that were PMs). The reason for this triggering of CYP2D6 expression at birth remains unclear.

Neonates up to 28 days of age have approximately 25% of adult levels of CYP2D6 protein and this continues to increase with increasing age. Between 28 days and 5 years levels approach 50% of those in adults. In all the younger age groups large interindividual variations exist which is consistent with the variations seen in adult populations and is probably due to the variations in genetic expression and the likely presence of some PMs.

Liver microsomes from these studies were also used to assess the O-demethylation of dextromethorphan to dextrorphan. Dextromethorphan is used as a probe drug for CYP2D6 as the O-demethylation reaction has been shown to be genetically controlled and it co-segregates with codeine O-demethylation.(Mortimer O et al 1990; Dayer P et al 1988) In fetal liver samples average O-demethylation activity was 1% of adult levels and was only present in a small proportion of the fetal samples. Activity rose within the first week of life but in the groups aged from 8 days to 5 years the activities were identical and were only 25% of adult levels. (figure 2)

No correlation was found between dextrorphan formation and CYP2D6 protein content when all the samples were analysed together. However, when three groups, fetuses, newborns and adults, were examined separately there was a positive correlation with age. This suggests that the protein is more efficient in carrying out the O-demethylation reaction in adults than neonates and fetuses. Since only one gene is expressed in the human genome these changes in catalytic activity with age might be due to minor changes in protein conformation and/or affinity for the substrate. The activity of membrane-bound enzymes is dependent on the phospholidid composition of the membrane. This has been shown to change between fetal and adult liver microsomes in both humans and rats.(Kapitulnik J et al 1986) Thus the catalytic activity may be changed either directly by changes in the protein conformation or indirectly via differences in substrate accessibility or changes in electron transfer from the NADPH cytochrome P450 reductase.

Dextromethorphan also undergoes N-demethylation to methoxymorphinan and can be used as a probe drug for N-demethylation reactions such as the conversion of codeine to norcodeine. In the studies discussed the N-demethylation reaction exhibited a different pattern of evolution. Activity was present in all fetal samples and remained stable in all fetal, neonatal and infant groups at about 20 –30% of adult activity. This confirms the fact that a different enzyme is responsible for this metabolic pathway and its development follows a different time course. These studies showed that the N-demethylation reaction was dependent on the CYP3A subfamily.

1.2.6 Rat models of CYP2D6

Genetics

The cytochromes P450 CYP2D6 and CYP2D1 subserve similar functions in man and rat respectively.(Matsunaga E et al 1989; Nebert DW et al 1989) In the Sprague Dawley (SD) rat the CYP2D1 enzyme is mainly responsible for the metabolism of substances affected by the sparteine/debrisoquine type polymorphism such as codeine and the female SD rat is used as an animal model for the human EM. The Dark Agouti (DA) rat lacks genetic expression for CYP2D1 which leads to a genetic deficiency of the enzyme and the female DA is used as an animal model of the human PM.(Boobis AR et al 1986).(Matsunaga E et al 1989) (Cleary J et al 1994; Mikus G et al 1991a)

CYP2D6 is the only isoform of the CYP2D subfamily present in humans. However, in rats five isoforms of CYP2D have been identified, CYP2D1-5. (Kawashima H et al 1995; Matsunaga E et al 1990; Gonzalez FJ et al 1987) Though the physiological significance of each isoform is not known it has been suggested that the low levels of CYP2D2 in DA rats is in part responsible for the decreased debrisoquine 4-hydroxylase activity.(Yamamoto Y et al 1998) These isoforms have also been identified in the brain and CYP2D has been shown to have catalytic activity in the brain.(Tyndale RF et al 1999) Both CYP2D2 and CYP2D5 have been suggested as the human CYP2D6 homologue though other studies and more recent work looking at dextromethorphan O-demethylase activity suggests that CYP2D1 is the main contender for the human CYP2D6 homologue in the liver and the brain.(Miksys S et al 2000; Tyndale RF et al 1999; Yamamoto Y et al 1998; Riedl AG et al 1997; Wan J et al 1997; Yamamoto Y et al 1996; Wu D et al 1993; Matsunaga E et al 1989)

No sex related differences in the drug metabolising enzyme activity have been established in humans.(Kato R et al 1982; Kremers P et al 1981) However, in rats, sex related differences are well known. Studies have indicated the presence of male and female forms of cytochromes P450 in rat livers and that the total cytochrome P450 content is different between adult male and female rats.(Matsubara T et al 1986; Kamataki T et al 1983; Kamataki T et al 1982; Kato R 1974) A study looking at cytochrome P450 monooxygenase activities in rat livers showed a significant difference between activities in adult male and female SD rats.(Matsubara T et al 1986)

The roles of CYP2D6 and CYP2D1 in drug metabolism in humans and rats respectively are not identical and care must be taken when extrapolating between the two species. The role of the female DA rat as a model for human PMs has been questioned. Some studies have suggested that CYP2D1 deficiency may be affected by age and substrate concentration and that deficiencies in oxidation may not be restricted to this isozyme, also that the handling of some compounds may be different in rats compared with humans and may thus involve other cytochromes P450.(Barham HM et al 1994; Mikus G et al 1991b; Vincent-Viry M et al 1988; Boobis AR et al 1986; Khan GC et al 1985) However, the evidence that the O-demethylation of codeine to morphine is impaired in female DA rats and that this has a detrimental effect on the analgesic benefit received from codeine is good in studies comparing DA with SD rats.(Cleary J et al 1994; Mikus G et al 1991a) The use of inhibitors such as quinine in SD rats confirms the role of O-demethylation in the analgesic effect of codeine and the role of CYP2D1 in its metaolism.(Cleary J et al 1994; Mikus G et al 1991a) A more recent study looked at the O-demethylation of codeine to morphine in rats using antiCYP2D1 antibodies found that around 80% of the O-demethylation reaction could be inhibited by use of high concentrations of the antibody indicating that this isozyme is mainly responsible for the conversion of codeine to morphine. The authors also found that antiCYP2C11 and antiCYP3A2 antibodies also inhibited the O-demethylation reaction to a minor extent and thus they maybe of some importance in the O-demethylation reaction in rats, though this has yet to be confirmed in vivo.(Xu BQ et al 1997)

Development

The influence of development on the cytochromes P450 in rats has not been widely studied. A study using female SD and Wistar (both counterparts of human EMs) rat liver homogenates at different developmental ages and looking at monooxygenase activity showed that, like humans, fetal O-demethylase acivity was very low and that activity increased markedly after birth. However, postnatally the situation was different in the rats. The O-demethylase activity was low initially but rose very quickly to reach a peak around P 20-30. The activity then decreased sharply to reach an intermediate level by P40-50 where it stayed throughout adult ages (figure 3).(Matsubara T et al 1986)

A more recent study has looked at developmental changes in the catalytic activity and expression of CYP2D isoforms in the rat using Wistar rats.(Chow T et al 1998) Liver microsomes were prepared from female rats aged 1, 3, 7, 14 and 40 weeks. Levels of CYP2D protein decreased slightly from week 1 to week 3 before increasing to a peak at week 14. From there levels decreased to an intermediate point at week 40. Activity was low at weeks 1 and 3 and then increased to reach a peak at week 7. Activity then decreased through weeks 14 and 40. With a low substrate concentration activity remained low and constant throughout the age groups. The mRNA levels of CYP2D1 and 2 remained constant throughout the age groups though the mRNA levels of CYP2D3 increased up to 14 weeks and this was thought to be responsible for the increase in CYP2D protein. Since the physiological function of each of these isoforms is unknown it is difficult to relate these findings to the developmental changes in activity or the effect they may have on codeine O-demethylation. To my knowledge there has been no work done looking at the influence of development in SD rats on the activity of the CYP2D1 enzyme in isolation or on the potential differences in codeine O-demethylation to morphine and the analgesic efficacy of codeine.

Similarly there has been little work done looking at development and cytochrome P450 activity in the DA rat. As suggested above age was thought to have an effect on CYP2D1 deficiency. These differences involved animals of 15 weeks of age based on measurement of the debrisoquine metacolic ratio and suggested that animals at this age had higher levels of metabolic activity for debrisoquine.(Vincent-Viry M et al 1988) However, studies in isolated perfused liver of rats aged 10-32 weeks show a consistent decrease in metabolic activity for DA rats compared with Wistar rats.(Barham HM 1993) Thus the metabolic differences seen in the first study may arise from other influences such as variation in renal function. Further evidence refuting an effect of age on the CYP2D1 deficiency in DA rats comes from a further study, which shows that CYP2D1 mRNA was not found in 1 and 5 months old DA rats. (Matsunaga E et al 1989)

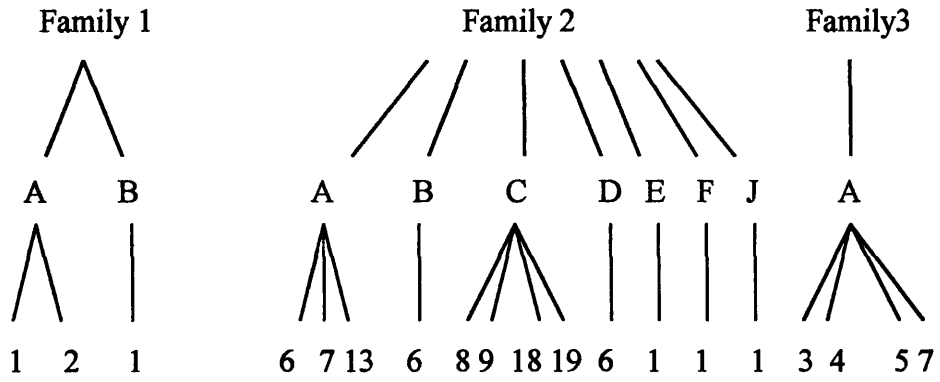

Figure 1. Human cytochrome P450 genes.

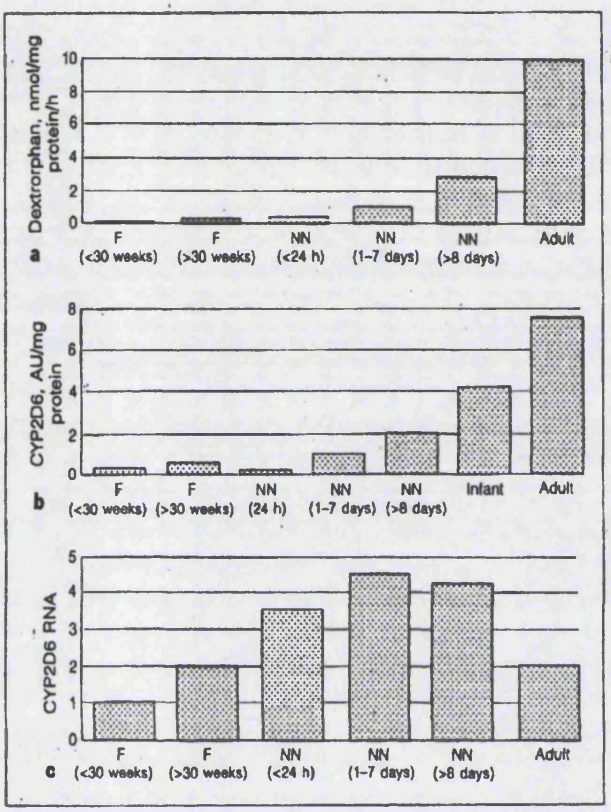

Figure 2. Age related variations of dextrorphan formation (a), CYP2D6 protein content (b) and CYP2D6 RNA (c) in human liver. F=fetus, NN=neonate, Infant=28 days –5 years. In (a) and (c) NN (>8days) = NN + Infant groups combined. From: Cytochrome P450-dependent metabolism of dextromethorphan: Fetal and adult studies. Jacqz-Aigrain E, Cresteil T. Developmental Pharmacology and Therapeutics 1992;18:161-168.

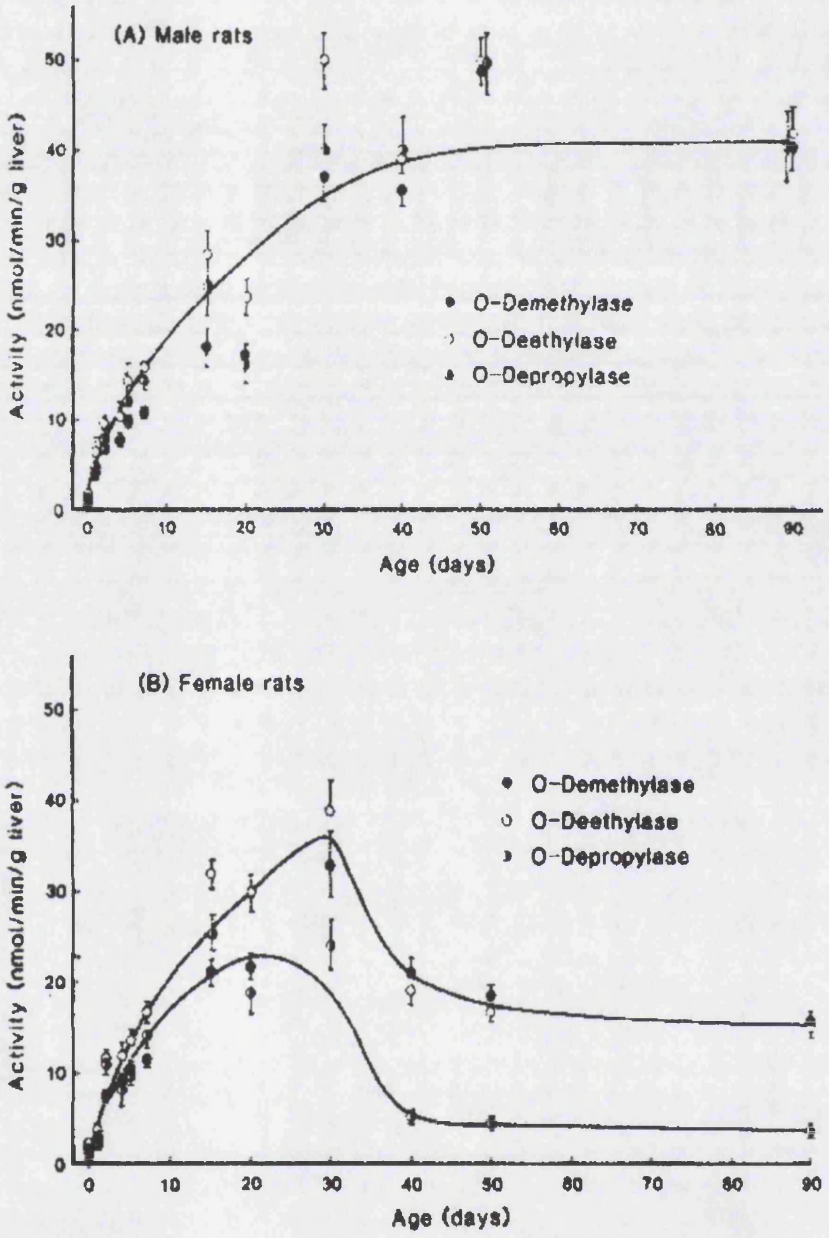

Figure 3. Developmental profile of 7-alkoxycoumarin O-dealkylase activities in rats. (A) Male rats, (B) Female rats. From: Cytochrome P-450-Dependent Monooxygenase Activities in Prenatal and Postnatal Human Livers: Comparison of Human Liver 7-Alkoxycoumarin O-Dealkylases with Rat Liver Enzymes. Mastubara T, Yamada N et al. Japan Journal of Pharmacology. 1986;40:389-398

1.3 Codeine Phosphate

Codeine is an old drug that still enjoys widespread clinical use although the logical basis for its popularity has been long questioned.(Lasagna 1964) It is considered to be suitable for mild to moderate pain but not for more intense pain even in large doses.(Reisime T et al 1996) The World Health Organisation has devised a 3-step analgesic ladder for the progressive treatment of increasing pain, on which codeine is considered a weak opioid and occupies a position on the second step (figure 4).(World Health Organisation. 1986) A significant degree of unpredictable, variable or poor response to treatment with codeine has been reported in many human and animal studies. Indeed, some single dose studies in adults have shown no difference between codeine and placebo.(Hoing S et al 1984; Jochimsen PR et al 1978) A quantitative systemic review suggests that codeine 60 mg has a number needed to treat (NNT) of 18 which is very high when compared with 5.0 for paracetamol 600 mg and 3.1 for the combination.(Moore A et al 1997)

Codeine is frequently recommended for paediatric use.(Dolhery C 1999; Prevention and Control of Pain in Children 1997; Schechter NL et al 1993) A recent survey of paediatric anaesthetists in the United Kingdom showed that alongside morphine and fentanyl, codeine is the most widely prescribed opioid analgesic in paediatric anaesthetic practice.(de-Lima J et al 1996) The reputedly lower incidence of opioid - related side effects has made codeine popular for the younger age groups including neonates and especially in situations where airway management and neurological assessment are critical.(Semple D et al 1999; Husband AD et al 1996; Stoneham MD et al 1995; Hatch DJ et al 1995; Lloyd-Thomas AR 1990) These suggested benefits have been noted after single doses though may not exist when repeated doses are used. (Lloyd-Thomas AR 1990) In fact, few clinical studies of the analgesic efficacy or side effects of codeine in children have been undertaken, and although the incidence of side effects may be low, analgesia may be inadequate for postoperative pain in some circumstances.(Semple D et al 1999) Pain assessment is difficult in paediatric populations especially neonates and preverbal children and this complicates both the study and use of analgesics particularly those with low efficacy or unpredictable effects. Significant variability in both the pharmacokinetics and pharmacodynamics of codeine has been shown in animal and adult human laboratory experimental studies.(Wilder-

Smith CH et al 1998; Eckhardt K et al 1997; Cleary J et al 1994; Mikus G et al 1991; Yue QY et al 1991; Chen ZR et al 1991a; Sindrup SH et al 1990) Both genetic and developmental factors may contribute to this variability in children although the clinical consequences are not known.

1.3.1 Pharmacology of codeine (Dolhery C 1999; Sindrup SH et al 1995)

Codeine is a naturally occurring opium alkaloid: 7,8 didehydro-4,5epoxy-3-methoxy-*17*-methyl-morphinan-6-ol monohydrate.(Martindale The Complete Drug Reference 1996) (Figure 5) Like morphine it is a constituent of the opium poppy, *Papaver somniferum.* It was isolated from opium in 1833 by Robiquet and its pain-relieving effects were recognised shortly after. Codeine constitutes about 0.5% of opium, which continues to be a useful source of its production, although the bulk of codeine used medicinally is prepared by the methylation of morphine. Codeine is less potent than morphine, with a potency ratio of 1:10.(Wallenstein SL et al 1961)

Dosage and Uses

Codeine can be given by the oral, rectal and intramuscular routes. The intravenous route is not recommended due to dangerous hypotensive effects probably related to histamine release.(Parke TJ et al 1992) In children, it is generally given in doses of 1 mg kg^{-1} up to a maximum of 3 mg $kg^{-1}day^{-1}$, though larger doses have been used.(British National Formulary 1999; Semple D et al 1999)

Codeine is often used in combination with other drugs e.g. aspirin, paracetamol, non-steroidal anti-inflammatory drugs (NSAIDs) and diphenhydramine in the treatment of mild to moderate pain. In neonates and children it has been used in both acute and chronic painful conditions and particularly for postoperative and cancer pain.(de-Lima J et al 1996; Hatch DJ et al 1995; Houck CS et al 1994; Schechter NL et al 1993; Lloyd-Thomas AR 1990)Its antitussive and constipating properties also mean that it is used in many preparatory cough, cold and antidiarrhoeal remedies.

1.3.2 Pharmacokinetics (Dolhery C 1999)

The vast majority of pharmacokinetic data for codeine have been obtained from adult investigations. Very little information is available from studies in children or infants and to my knowledge there is no published work in neonates.

Codeine is rapidly and well absorbed following oral administration, approximately 50% undergoing pre-systemic metabolism in the gut and liver. Peak plasma concentration occurs after approximately 1 hour and the plasma half-life is 3-3.5 hrs. Absorption is faster after intramuscular injection, the time to peak plasma concentration being about 0.5 hrs. The volume of distribution is 3.6 l kg^{-1} and the clearance is 0.85 l min^{-1}.

The oral:intramuscular potency ratio is 1:1.5 and implies that the oral bioavailability is greater than that of morphine. Codeine is distributed rapidly to the tissues and is concentrated in the liver, kidney, lung and spleen, though the bulk of the total drug remains in the skeletal muscle. Codeine does not accumulate in the brain, with over 80% of that in the brain being associated with opioid receptors. Codeine also crosses the placenta and can be found in the milk of lactating women.

After a normal therapeutic dose of codeine the plasma concentration is in the range of 100 – 300 $mcgl^{-1}$. Excretion occurs mainly in the urine (86%), of which 5 - 15 % is free drug. A small amount is excreted in bile and faeces.

Rectal codeine has been recently introduced into paediatric practice. An earlier study in healthy adult volunteers showed no difference in codeine bioavailability following rectal or oral administration with a systemic availability of about 90%.(Moolenaar F et al 1983) In the postoperative period these pharmacokinetic values may be less predictable. One study found that clearance varied four-fold and systemic availability after oral dosage was between 12 – 84 %.(Persson K et al 1992)

Available research findings imply that age specific differences in the pharmacokinetics of codeine may be significant. In a comparison of intramuscular and rectal codeine for postoperative analgesia in children aged three months to 12 years, peak plasma levels of codeine were achieved, as expected, between 30 and 60 minutes in both groups but

rectal bioavailability was found to be lower.(McEwan A et al 2000) In another study of rectal codeine for postoperative analgesia in infants and children aged between 6 months and 4 years, the mean initial half-life was 2.6 hours, but in the infants of the lowest body weight, the half life was over 2 hours longer than this mean value.(Quiding H et al 1992a) In addition, plasma drug concentration data indicate that a rectal dose of codeine of 0.5 mg kg^{-1} in children can result in similar, or slightly higher, plasma concentrations of codeine and its metabolites than after 60 mg orally in adults.(Quiding H et al 1986) More information is clearly required, particularly for neonates and in the postoperative setting in order to understand fully the effects of development on codeine pharmacokinetics in clinical situations.

Metabolism(Dolhery C 1999)

Codeine is principally metabolised in the liver in one of three ways: glucuronidation at the 6-OH position, the principal route; N-demethylation to norcodeine (10 – 20%); and O-demethylation to morphine (5 – 15%). Between 5 –15% of the drug is excreted unchanged in the urine (Figure 6). Other minor metabolites, normorphine and hydrocodone, have also been identified.(Martindale The Complete Drug Reference 1996)

In 1948, Sanfilippo first suggested that the analgesic effect of codeine was due to the proportion of the drug metabolised to morphine and this has lead to the belief, now supported bt subsequent evidence, that codeine is a prodrug, with morphine as its principal active metabolite and having little or no intrinsic analgesic activity of its own.(Sanfilippo G 1948)

The efficacy of a prodrug is dependent on the amount of active metabolite formed. Variable expression of the enzymes involved in the biotransformation of drugs can lead to substantial differences in the production rate and plasma concentration of metabolites and hence the efficacy of a prodrug.

Variation in drug metabolism can have a number of causes:

- Genetic polymorphisms – genetic variants of the enzyme responsible for metabolism resulting in different metabolising capacities.
- Induction or inhibition due to concomitant drug therapies or environmental factors.
- Physiological status - including development.
- Disease states.

It has been known for some time that genetic variability in drug metabolism is an important cause of inter-individual variations in drug efficacy. Maturation of enzyme systems is another important factor for certain compounds. Both of these may have an effect on the efficacy of codeine.

Genetic Variation

The O-demethylation of codeine to morphine is dependent on the cytochrome P450 enzyme, CYP2D6 which is known to show genetic polymorphism. The other metabolic pathways for codeine are not dependent on this enzyme.(Yue QY et al 1989; Chen ZR et al 1988; Dayer P et al 1988) N-demethylation for example is catalysed by CYP3A, another P450 enzyme.(Yue QY et al 1997; Hedenmalm K et al 1997)

A large number of different genetic variants are known to exist for CYP2D6 which leads to a wide spectrum of metabolic capabilities within study populations.(Marez D et al 1997; Daly AK et al 1996) When discussing these differences in metabolic capability, individuals are normally classified as either PMs or EMs depending on the activity of the enzyme, although this is known to be an oversimplification.(Gaedigk A et al 1991; Heim M et al 1990; Kagimoto M et al 1990; Zanger UM et al 1988) Poor metabolisers will produce little or no morphine from codeine whereas extensive metabolisers will produce significant amounts of morphine although the actual amount produced may show wide variation.(Sindrup SH et al 1990; Rogers JF et al 1982; Findlay JWA et al 1978) These metabolic differences between PMs and EMs are known to remain constant even after long term codeine administration.(Yue QY et al 1991; Chen ZR et al 1991a)

Development

Despite the popularity of codeine in paediatric practice, the influence of development on the efficacy and side-effects of codeine has not been well investigated. It has been suggested that infants and neonates have a reduced metabolic capacity for codeine.(Quiding H et al 1992a) CYP2D6 activity is absent or less than 1% of adult values in fetal liver microsomes. O-demethylation of codeine to morphine does not occur *in utero* but activity of the enzyme is known to increase markedly immediately after birth regardless of gestational age. This increase in enzyme activity is maintained, although it may still be below 25% - 50% of adult values at five years of age. The efficiency of the O-demethylation reaction also appears to be much lower in neonates than in adults. The glucuronidation pathway is also immature at birth and it has been shown that it continues to develop after the neonatal period. In contrast, N-demethylation has been found to be equivalent to that in adults at all pre and post natal ages.(Jacqz-Aigrain E et al 1992; McRorie TI et al 1992; Ladona MG et al 1991; Treluyer J-M et al 1991; Ladona MG et al 1989)

1.3.3 CYP2D6 and codeine - pharmacokinetics

It has been known for many years that following codeine intake some individuals had very low plasma levels of morphine and it was suggested that this was due to genetic polymorphism in the O-demethylation of codeine.(Rogers JF et al 1982; Findlay JWA et al 1978) Pharmacokinetic studies have since confirmed that CYP2D6 is an important enzyme catalysing the formation of morphine from codeine.(Yue QY et al 1989; Chen ZR et al 1988; Dayer P et al 1988) DA rats, which lack the equivalent of the CYP2D6 enzyme, are an animal model for PMs and have been shown to have different pharmacokinetics for codeine O-demethylation compared to SD rats, which do possess the equivalent enzyme and act as controls.(Mikus G et al 1991)

A study using human adult volunteers, took 12 EMs and 12 PMs and looked at plasma morphine levels following an oral dose of 75mg of codeine. Morphine was not detected

in any of the PMs, whereas in the EM group 11 had morphine levels ranging from 4.9 – 37.6 $nmoll^{-1}$ and 1 was below the quantification level.(Sindrup SH et al 1990) Further experimental work in adult humans showed that morphine and its metabolites were detected in only one of the PMs and then at much lower levels, by up to a factor of 20, than those detected in the EMs.(Yue QY et al 1991; Chen ZR et al 1991a) In these two latter studies there was a phase of chronic codeine dosing in which the pharmacokinetic differences between the PMs and EMs remained unchanged. In all three studies there was no significant difference between the PM and EM groups with regard to codeine and its non O-demethylated metabolites.

Human studies of codeine metabolism have been refined by the pharmacological blockade of the CYP2D6 enzyme. This was achieved by using a substrate that had a relatively greater affinity for the CYP2D6 enzyme than codeine, such as quinidine. These studies showed an apparent blockade of O-demethylation in EM subjects by pretreating them with quinidine.(Sindrup SH et al 1992; Desmeules J et al 1991) In the latter study the morphine level in plasma ranged from 6 – 62$nmoll^{-1}$ when codeine was given alone but was below the level of detection in the entire group when they were pretreated with quinidine. Codeine has a relatively low level of affinity for CYP2D6 and hence competitive interaction is likely to occur when codeine is combined with other substrates or inhibitors of CYP2D6.(Dayer P et al 1992; Dayer P et al 1988) Other drugs such as NSAIDs, benzodiazepines and anticonvulsants, which are not substrates for this reaction, do not influence the reaction.

A more recent study looked at the urinary excretion of codeine and all its metabolites after 25mg of codeine was given to 18 PMs, 114 EMs and 24 ultra-rapid EMs. The recovery of the O-demethylated metabolites showed significant variation between the groups. In the PMs it was less than 0.4%, for the EMs it was 1.7 – 8.7 % and for the ultra-rapid EMs 15.3%. The conclusion was that ultra-rapid EMs may potentially develop increased O-demethylated metabolite dependent effects or side effects of codeine.(Yue QY et al 1997)

1.3.4 Pharmacodynamics

Mechanism of action

Potentially codeine could act via a number of different mechanisms. It may have a direct (opioid or non-opioid receptor mediated) analgesic effect or it may act through metabolism to morphine or other active metabolites. Codeine binds to the mu receptor like morphine but with a much lower affinity, by a factor of between 200 - 600.(Raffa RB et al 1993; Chen ZR et al 1991b; Neil A 1984; Pert CB et al 1973) It also binds to the kappa and delta receptors but again has a much lower affinity than morphine, though the difference is less marked.(Raffa RB et al 1993; Neil A 1984) The affinities of the metabolites codeine-6-glucuronide and norcodeine to the mu receptor are similarly low.(Irvine RJ et al 1989) Early animal studies produced conflicting results; morphine tolerant mice were found to be less tolerant of codeine and the analgesic effect of codeine is less easily reversed by naloxone than that of morphine.(Neil A 1982; Alder TK 1963) Although, in a rat model significant analgesia was demonstrated only in those animals able to metabolise codeine to morphine.(Cleary J et al 1994)

Further attempts to elucidate the mechanism of action of codeine used adult human experimental models to compare the effect codeine on phasic and tonic pain. Phasic pain is due to a stimulus of varying intensity, analogous to pain with movement, which is known to respond poorly to opioids. Tonic pain due to a constant intensity stimulus, is analogous to rest pain and is opioid sensitive.(Cooper BY et al 1986; Eriksson MBE et al 1982; Beecher HK 1957) In this study codeine and morphine were shown to have no effect on opioid-insensitive phasic pain in either extensive or poor metabolisers, which suggests that a non-opioid receptor mediated effect for codeine is unlikely. Results from this study also showed that for codeine EMs but not PMs had a good response for tonic pain whereas both EMs and PMs had a similarly good response to morphine, suggesting that in man codeine analgesia is both an opioid effect and that the formation of morphine is an important factor.(Poulsen L et al 1996)

Experimental efficacy

The discovery of CYP2D6 and its critical role in the conversion of codeine to morphine allows the analgesic effect of codeine in animals and humans to be examined in known PMs and EMs i.e. in situations of extensive or minimal production of morphine.

Using adult human volunteers it was shown that 75mg of oral codeine increased pain thresholds to cutaneous high energy laser stimulation in EMs but not PMs and that these increases in thresholds correlated with plasma levels of morphine.(Sindrup SH et al 1990) Further work using a rat animal model compared the effect of codeine on tail flick latency in DA rats, which cannot O-demethylate codeine, with that of control SD rats. There was no effect of codeine administration on tail flick latency in the DA rats but there was a clear increase in latency in the control rats.(Cleary J et al 1994)

Confirmatory evidence for the dominant role of morphine in the effects of codeine in man have been obtained by competitive inhibition of codeine O-demethylation using quinidine.(Sindrup SH et al 1992; Dayer P et al 1992; Desmeules J et al 1991; Dayer P et al 1988) The results from these studies show that codeine analgesia in extensive metabolisers could be blocked by pretreatment with quinidine. Studies in (adult human) volunteers using the same technique have shown that the incidence and magnitude of respiratory, pupillary and psychomotor effects are also dependent on codeine metabolism to morphine.(Caraco Y et al 1997; Caraco Y et al 1996)

More recent work has also shown that, even at high doses, no analgesic effect from codeine could be demonstrated in PM groups. However in EM group, analgesia was found to be slightly greater following codeine than with a dose of morphine producing similar plasma levels of morphine.(Eckhardt K et al 1997) Although this work provides strong evidence for the dominant role of morphine in codeine analgesia, the slightly increased efficacy of codeine at similar plasma levels of morphine must be explained. Several possibilities have been suggested:

1. A small analgesic effect of codeine itself or the other metabolites . A recent study using a rat animal model has shown that hydrocodone, a possible minor codeine metabolite, has analgesic properties which are independent of its O-demethylated metabolite hydromorphone.(Tomkins DM et al 1997) However, the lack of analgesic

effect consistently seen in poor metabolisers makes both this mechanism and a direct effect of codeine itself unlikely or only a very small component of the pharmacodynamic effect.

2. Another explanation may be that the rapid penetration of the blood brain barrier (BBB) by codeine and expression of CYP2D6 in the brain lead to higher levels of morphine in the central nervous system. Codeine is known to penetrate the BBB more rapidly than morphine,(Oldendorf WH et al 1972) and the presence of CYP2D6 in the CNS allows local tissue O-demethylation of codeine to morphine. This has been shown to be possible in rat brain homogenates and the presence of CYP2D6 has been demonstrated in both human and animal brain tissue.(Allard P et al 1994; Tyndale RF et al 1991; Niznik HB et al 1990; Chen ZR et al 1990; Lee EJD et al 1989; Fonne-Pfister R et al 1987) The fact that CYP2D6 is found in the CNS would also be compatible with the lack of analgesic effect of codeine in PMs since a deficiency of CYP2D6 would be expected to occur in all tissues, including the brain, if it was of genetic origin.

3. Demonstration of a direct correlation between analgesia and plasma levels of morphine or its metabolites has not been possible and studies difficult to interpret, particularly in children. (Millar AJW et al 1987; Lynn AM et al 1984; Nahata MC et al 1984; Dahlstrom B et al 1979) This may suggest that local factors within the CNS such as morphine levels, receptor site morphine levels, receptor number and concentrations and receptor binding may be more important than plasma morphine levels when looking at efficacy.

Clinical Efficacy

Both adult and paediatric clinical studies have demonstrated that the efficacy of codeine is low, and that it has a ceiling effect at higher doses above which there is a marked increase in the incidence of side-effects.(McEwan A et al 2000; Semple D et al 1999; Quiding H et al 1993; Persson K et al 1992; Lloyd-Thomas AR 1990) Two recent systematic reviews in adults have shown a small but significant benefit of adding codeine 60 mg to paracetamol.(Moore A et al 1997; de Craen AJM et al 1996) Many

other studies, also in adults, confirm that codeine 30–60 mg can add significantly to the analgesic effect of drugs such as aspirin, paracetamol and NSAIDs.(Quiding H et al 1992b; Arendt-Nielsen L et al 1991; Kjaersgaard-Andersen P et al 1990; Happonen R-P et al 1987; Dahl E et al 1985; Cooper SA et al 1982; Forbes JA et al 1982) This additive effect is especially prominent after repeated doses, and accumulation of morphine may be an explanation.(Quiding H et al 1992b; Giles ED et al 1985)

There have been very few clinical studies investigating the analgesic efficacy of codeine in which genetic variation is taken into consideration in adults, and none in paediatric groups. In the only study of postoperative patients to consider this factor, the analgesic efficacy of codeine was found to be low but overall there was no difference in efficacy between the two phenotypes (EM and PM).(Poulsen L et al 1998) However, the number of PMs was very small and the overall efficacy of codeine was low throughout the study, with 41% of patients requiring escape analgesia. Also, amongst the EMs there was a very wide spectrum of metabolic ability and patients with higher concentrations of morphine and morphine-6-glucuronide had a significantly better analgesic response. Interestingly, serum concentrations of morphine and its metabolites after 1 hour were much lower in the EMs in this study than those which have been generally found in healthy EM volunteers.(Sindrup SH et al 1992; Yue QY et al 1991; Desmeules J et al 1991; Chen ZR et al 1991a; Sindrup SH et al 1990; Quiding H et al 1986) This suggests that postoperative factors may also significantly influence codeine metabolism, affecting the formation of morphine and its metabolites.

Non-analgesic Effects

Codeine has a reported side-effect profile which is broadly similar to other opioids:

- Respiratory depression
- Sedation
- Nausea and vomiting
- Decreased gastric motility
- Miosis

- Dependence, though this is markedly less than with morphine
- Large doses cause excitation rather than mood depression
- Hypotension when given by the intravenous route (Parke TJ et al 1992)

One of the reasons codeine has maintained its popularity is a reputedly lower incidence of these side effects in comparison with other drugs from this group. Codeine is commonly used for postoperative analgesia following neurosurgery due to its supposed lack of sedation, respiratory depression and the preservation of pupillary signs. A recent survey of adult neuroanaesthetists suggested that codeine and dihydrocodeine were the mainstays of postoperative analgesia, but in 50% of cases the same anaesthetists considered this analgesia to be inadequate. Most said they would not use morphine as an alternative for fear of side-effects.(Stoneham MD et al 1995) There is little evidence to confirm that the side-effects of codeine are significantly fewer or less severe than those of other opioids at equi-analgesic doses. The antitussive and gastrointestinal (GI) effects of codeine have been found to occur at lower doses than those for analgesia, but the mechanism has not been fully elucidated.(Sindrup SH et al 1995)

Studies of GI transit time in adults have been largely inconclusive. Two studies comparing the effect of codeine on GI transit in PMs and EMs showed no significant difference between the two groups.(Hasselstrom J et al 1997; Hasselstrom J 1992).However, another study looking at this question in further groups of PMs and EMs and measuring plasma codeine and morphine levels, found that both groups had equal plasma codeine concentrations but that the EM group had significantly higher plasma morphine concentrations and longer transit times.(Mikus G et al 1997)

The extent to which the different non-analgesic and adverse effects of codeine can be attributed to metabolism to morphine is not known; there is increasing evidence, however, of a direct role for codeine itself. Single dose comparisons of codeine, morphine and placebo in adult volunteers have shown that at low doses side-effects appear to be directly related to plasma morphine levels but that at higher doses codeine may itself be directly responsible for adverse effects as the incidence of adverse effects was equivalent in both the EM and the PM groups.(Eckhardt K et al 1997; Poulsen L et

al 1996) The importance of this finding is that, ironically, PM groups whilst having inadequate analgesia may still experience side-effects from codeine.

Another group of patients that may experience enhanced adverse effects from codeine are the ultra-rapid extensive metabolisers. Recovery of the O-demethylated metabolites is much higher in the ultra-rapid EMs (15.3% versus 1.7 -8.7% in EMs and 0.4% in PMs).(Yue QY et al 1997)Thus ultra-rapid EMs may develop increased morphine dependent effects or side effects of codeine and in addition lower than normal doses may be necessary to achieve the required therapeutic effect.(Bertilsson L et al 1993; Bertilsson L et al 1985) In an interesting case report, a 33 year old woman experienced the acute onset (less than 30 minutes) of colicky abdominal pain, euphoria and dizziness following codeine 60 mg after a tooth extraction.(Dalen P et al 1998) The same symptoms recurred following a second dose of 30 mg. She was later phenotyped and found to be an ultra-rapid EM. Rapid and extensive formation of morphine by O-demethylation of codeine was the suggested cause of these symptoms.

Absence of or reduction in unpleasant or potentially dangerous side-effects for equal analgesia in comparison with other opioids would be an important advantage for codeine. However, there is little evidence for this. It has been suggested that a dose of codeine 1 mg kg^{-1} intramuscularly or orally in neonates and children is associated with a low risk of respiratory depression, though with repeated dosing this advantage may be lost.(Reisime T et al 1996) In fact, there are little data on the non-analgesic effects of codeine in children. Respiratory and pupillary effects in comparison with other opioids have not been specifically investigated.

Post operative vomiting after adenotonsillectomy in children has been found to be significantly less after codeine in comparison with morphine for 'equipotent' doses in a recent study.(Semple D et al 1999). They found a 60% vomiting rate in the morphine group compared with 30 % in the codeine group. Again, however, this study did not take genetic variability into consideration.

1.3.5 Conclusion

As the major proportion of the analgesic effect of codeine appears to be due to its metabolism to morphine a large interindividual variation in efficacy is to be expected with possibly approximately 9% of the UK population having little or no benefit. In effect, codeine administration could possibly be regarded as a complicated and unreliable way of giving a low dose of morphine. As long ago as 1964, Lasagna noted that "codeine possessed most of the disadvantages of morphine" and that "its use could be avoided by using a dose of morphine less than 10 mg!".(Lasagna 1964) Pharmacokinetic effects, particularly in the postoperative period, may also reduce its efficacy.

In early life, the immaturity of infant metabolic processes is also likely to reduce the efficacy of codeine. Furthermore, there is evidence that adverse effects may occur in the absence of analgesia in poor metabolisers of the drug. Genetic polymorphism may be an important factor responsible for the low efficacy of the drug in population studies and the high NNT in systematic reviews. It is interesting to note that during drug development those compounds subject to selective metabolism by polymorphic enzymes are often discarded in the early stages.(Ingleman-Sundberg M et al 1999) If codeine had been discovered in the present day it is possible that it would not have made it as far as the pharmacy shelves.

The development of efficient and inexpensive methods of genotyping for enzyme polymorphisms, transport proteins and drug receptors may enable genetic information to be used in the future to tailor drug therapy for each individual.(Ingleman-Sundberg M et al 1999) However, until this type of screening is widely available, there is no way to accurately predict whether an individual patient will obtain a significant analgesic effect from a dose of codeine.

Overall there has been little clinical research into the use of codeine in children. From that which has been done it appears that the popularity of codeine in this patient group is not supported by convincing data of its efficacy or suitability, despite its apparently good safety record. The available evidence implies that codeine may in fact be particularly unsuitable for use in the younger child given the difficulties in pain

reporting and assessment in this group and the unpredictable effects of the drug. In comparison with other opioids there is little evidence to support the commonly held view of a reduced incidence of serious side-effects in the presence of equivalent analgesia.

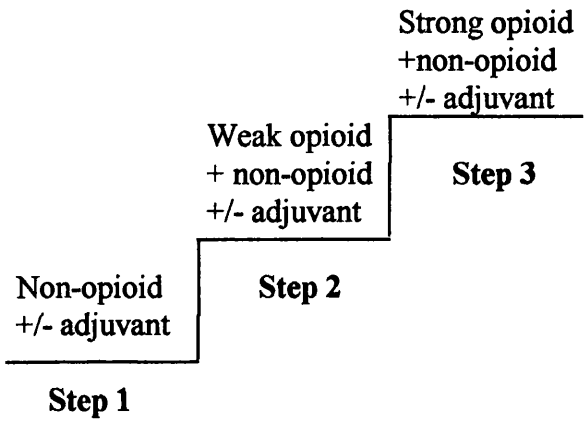

Figure 4. W.H.O. Analgesic Ladder

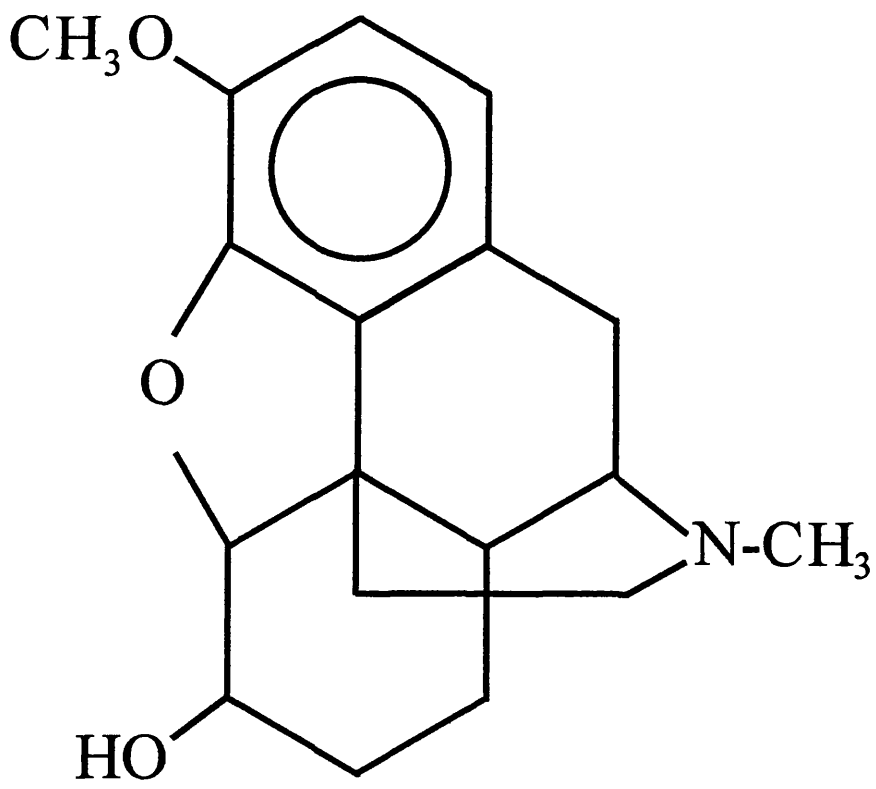

Figure 5,Codeine

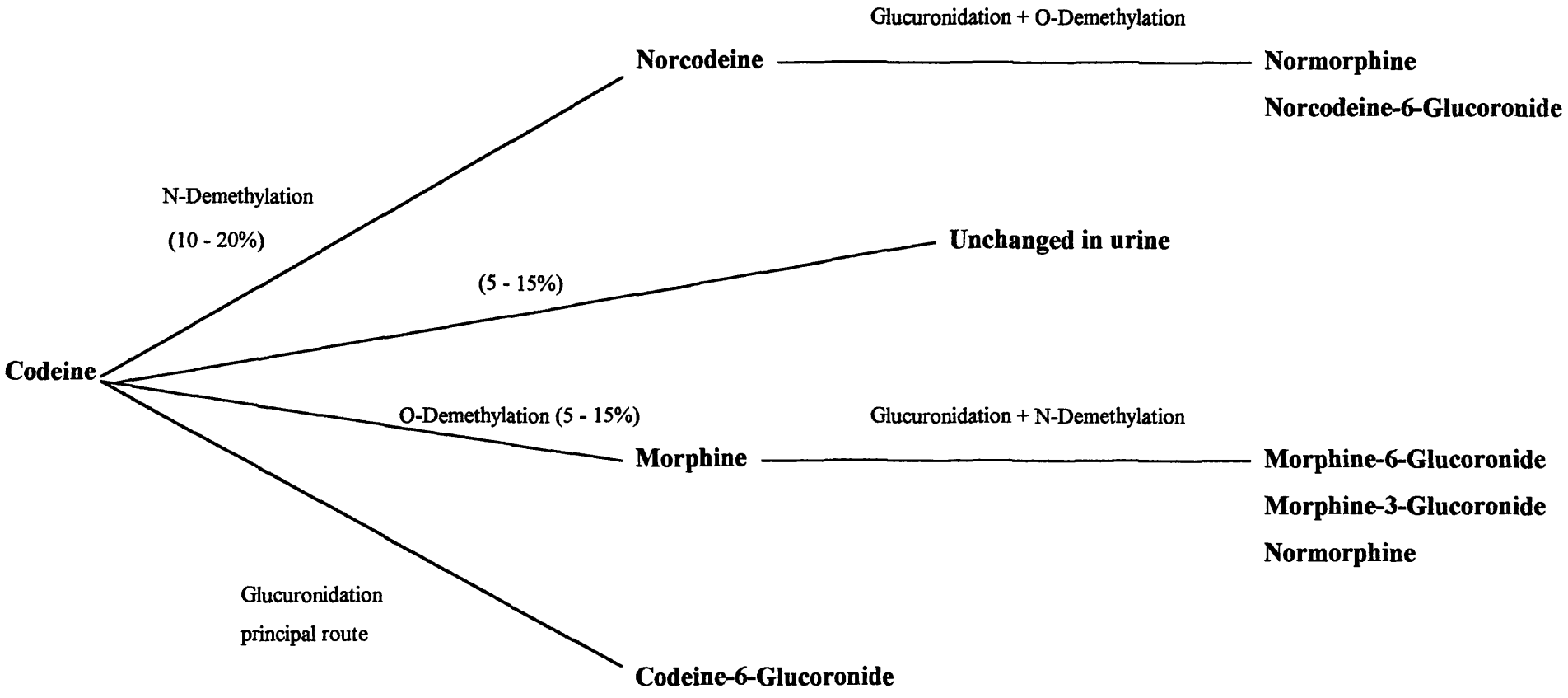

Figure 6. Metabolism of codeine

1.4 Aims of thesis

From the review of the literature presented in this Chapter it would seem that the available evidence suggests that the analgesic efficacy of codeine is variable and that the analgesic effect is either mostly or wholly dependent on the ability of the individual to convert codeine to morphine.

Codeine is considered an important weapon in the analgesic armoury of the paediatric aneasthetist, mainly due to the presumed benefit of good analgesia combined with a low incidence of opioid related side-effects. However, there is little in the available literature to support this assumption. In fact for younger children, such as neonates and infants, the evidence may suggest that there is potentially reduced efficacy. Pain assessment in these preverbal children is very difficult and thus it is vital to be confident of the reliability of the analgesia administered to ensure adequate treatment.

Two factors that may be responsible for variations in drug efficacy are genetic variation and development. The work in this thesis was designed to investigate these two areas, with relation to the efficacy of codeine, using both laboratory experiments and clinical study.

Laboratory experiments

These experiments encompassed a large proportion of the work in the thesis. Initial work was designed to look at the effect of development on the efficacy of codeine. From the literature we know that the activity of the CYP2D6 is reduced in the neonatal and infant periods and only reaches 25 - 50 % of adult levels by the age of five years. The question this raises is how much enzyme activity is required for analgesic efficacy? If there were low levels of enzyme activity would codeine still be effective? Attempts to answer this question involved the use of a behavioural studies using an animal model, the SD rat, which is known to be able to convert codeine to morphine. Animals were compared at different stages of neurological and metabolic development i.e. P3, P10, P21 and P63-70 (i.e. adult).

In the female SD rat the O-Demethylase activity of the rat is low at P3 but rises steeply in the early postnatal period to significantly higher levels by P10 and P21. A plateau of activity is reached around these ages which then declines to adult levels by P40-50, which is still higher than the levels of activity seen at P3. Activity then seems to remain constant through adult life.(Matsubara T et al 1986) The P3 SD rat was thus used as a model of low O-demethylase activity.

The sequence of events that takes place in the neurological development of the rat and human CNS are very similar but it is difficult to make exact comparisons. As a guide the P3 animal is said to equivalent to a 28 week foetus at birth, a P10 animal to an infant and a P21 animal to an older child or adolescent. Though these comparisons are only estimates and are the subject of continued debate. Rat CNS maturation is accelerated compared with humans and is mature by four weeks.(Fitzgerald M et al 1993)

Initial experiments involved the P3, P10 and P21 SD animals being treated with intra-peritoneal doses of either morphine or increasing doses of codeine and then subjected to a mechanical stimulus, using von Frey hairs, to produce dose response comparisons for codeine at each age and elucidate equi-potent doses of codeine and morphine for use in further experimentation.

The experiments using mechanical stimulation were also repeated using adult SD animals, to give a comparison between the adult and developing animal, and in DA rats, as an animal model of the poor metaboliser of codeine, at equivalent ages to look at the combined effects of development and genetic variation.

A hot water bath was then used to study the effects on SD animals treated with codeine and morphine of a thermal stimulus. These experiments involved the P3, P10 and P21 age groups.

The experiments involving mechanical and thermal stimuli were performed on animals that not been subjected to any prior intervention i.e. naive. Thus a further set of experiments was designed to test the effects of codeine and morphine on mechanical

stimulation in SD rats (P3, P10 and P21) in whom a localised inflammatory reaction had been induced using carrageenan to simulate tissue injury.

Experiments using mechanical stimulation and inflammation were repeated with each animal being given simultaneous injection of the opioid to be tested and naloxone, an opioid receptor antagonist, to ascertain that any anti-nociceptive effect being demonstrated was due to an opioid effect.

Plasma morphine and morphine metabolite levels can be used as an indirect measure of enzyme activity. Following on from the behavioural work, experiments were carried out involving blood sampling following injection of opioid or saline to allow for measurement of plasma levels of morphine and its glucuronide metabolies. These involved both SD and DA rats, again at the same ages i.e. P3, P10, P21 and adult, injected with the same doses of codeine and morphine.

Clinical study

The clinical study was designed to look at the role genetic variation plays in the efficacy of codeine. Tonsillectomy with or without adenoidectomy was chosen as the operative procedure to be studied due to:

- its regular performance in the paediatric population
- the fact that the analgesic regimen chosen usually includes codeine as the opioid of choice, since it is felt that the surgery is painful enough to require treatment with an opioid but that, due to the nature of the work being in the airway, sedation and respiratory depression are best kept to a minimum.

The children were randomised to receive either codeine or morphine as part of a fixed analgesic regimen. They were genotyped so EMs and PMs could be identified and they were assessed on the basis of pain scoring and plasma morphine levels so comparisons could be made between the different groups.

Hypotheses

1. The anti-nociceptive effect of codeine is an opioid effect and is due to metabolism of codeine to morphine.

2. There is developmental regulation of the efficacy of codeine

3. Reduced activity of the CYP2D6 enzyme is associated with reduced efficacy of codeine.

4. Poor metabolisers of codeine produce little or no morphine from a dose of codeine and thus have little or no analgesic effect from codeine.

CHAPTER 2. BEHAVIOURAL STUDIES

2.1 Materials and Methods

All rats were kept fed, watered and with their mother until the start of each experiment. Female SD rats were obtained from the animal house at University College, London. Experiments involved comparisons of animals aged P3 (mean weight 9.47g ±1.25), P10 (mean weight 23.05g ±3.11) and P21 (mean weight 48.81g ±6.46).

2.1.1 Injection techniques

In all the experiments the drugs were given via the intra-peritoneal (i.p.) route. The injections were administered with the animal awake and securely restrained, as anaesthesia may have compromised subsequent experimental recordings,. All drugs were diluted using sterile normal saline to give a volume of 10ml/kg to minimise any differences in absorption. This gave approximate volumes of 0.1ml at P3, 0.25ml at P10 and 0.5ml at P21. The injections were administered into the left lower quadrant of the abdomen using either a 0.5ml (insulin), 1ml or 2ml syringe, depending on the volume to be injected, and a 30gauge (G) needle .

2.1.2 Opioids studied

All the experiments involved comparisons between morphine sulphate and codeine phosphate. Morphine sulphate (University College Hospital Pharmacy) 1mg/ml and codeine phosphate (University College Hospital Pharmacy) 60mg/ml were diluted in sterile normal saline to achieve the required concentrations and volumes for experimentation.

Initial experiments were performed using SD P21 animals, exposed to mechanical stimulation, given morphine at doses of 1mg/kg, 2mg/kg and 4mg/kg. From the results obtained, a dose of 2mg/kg was shown to give a large reproducible effect with little sedative effect, this dose was subsequently used in experiments in all age groups. In

further experiments involving codeine and SD animals exposed to a mechanical stimulus the following doses were used:

SD P3 – 7.5, 10, 15 and 30mg/kg

SD P10 – 7.5, 10 and 15mg/kg

SD P21 – 7.5, 10 and 15mg/kg

From these results, it was not possible to find an exactly equipotent dose of codeine and morphine that were consistent across the different age groups. However, the nearest approximation was given by using morphine 2mg/kg and codeine 10mg/kg and thus these two doses were used in all subsequent experiments.

2.1.3 Cutaneous flexion withdrawal reflex

In each of the experiments the cutaneous flexion withdrawal reflex was used as the indication of a response to a stimulus. A withdrawal reflex is a response to a cutaneous stimulus that results in the removal of limbs from sources of potential tissue damage. It is a protective response involving coordinated muscle contractions at multiple joints through polysynaptic spinal pathways. The size and strength of the reflex muscle contraction is graded according to the intensity of the stimulus.

With flexion of the stimulated limb, extensor muscles groups in the opposite limb are excited and flexors inhibited thus enhancing the postural support when withdrawing from the noxious stimulus. The anatomical organization of the withdrawal reflex allows nociceptive cutaneous receptive fields to be linked to specific muscles and muscle groups such that the reflex results in the appropriate withdrawal of the area of stimulated skin.(Weng HR et al 1996) Each muscle has both inhibitory and excitatory cutaneous receptive fields suggesting that the reflex is organised in a modular fashion, each module being concerned with a particular muscle and thus the reflex can be thought of as an integrated series of parallel reflex pathways each subserving different muscles.(Schouenborg J et al 1995)

Cutaneous flexion withdrawal reflexes are not necessarily direct and absolute indicators of pain perception but they do provide information about the sensitivity and selectivity

of the nervous system to nociceptive stimuli. Any reflex response can be used as a measure of pain provided that it was elicited by stimulation of pain nerve endings only.(Goetzl Q 1943) Implicit in this is the assumption that the threshold of the withdrawal reflex is directly correlated with pain perception and this has been the subject of much debate.

The reflex has been used extensively to evaluate "antinociceptive" agents, with an increase in the threshold being interpreted as analgesic action. Its value has been shown in investigating spinal nociceptive processing and its association with post injury pain hypersensitvity and pain behaviours confirmed the withdrawal reflex as a powerful tool in pain research.(Woolf CJ 2001; Woolf CJ et al 1985; Woolf CJ 1983) It is a widely accepted simple experimental method that is non-invasive and can be used in awake subjects but its actions on motor neurons and sensory neurons could confound results.

Studies in human volunteers have shown pain thresholds almost identical to that which activates the flexion withdrawal reflex but that the magnitude of withdrawal does not correlate with the stimulus strength.(Campbell IG et al 1991; Willer JC 1977) However, these studies were carried out in controlled experimental conditions and as such may not be a reflection of the situation in "real life" and making generalizations in relating sensory thresholds to pain perception may be an oversimplification.(Wall PD 2000) Some forms of innocuous stimulation can evoke a withdrawal reflex, though the responses are weaker and nociceptors clearly provide the major input from skin to the reflex pathways. Skin inflammation and tissue injury can significantly increase the magnitude of a response to a normally innocuous stimulus and repeated thermal stimulation has been shown to reduce the threshold of nociceptors to thermal stimuli.(Hendry 1999)

Due to the fact that pain thresholds do not necessarily correlate with activation of the withdrawal reflex it may better to consider the reflex as a model of spinal nociceptive processing rather than of pain perception. It can be thought of as the nociceptive withdrawal reflex and considered in terms of its basic anatomy and function:

- peripheral nociceptors and nociceptive primary afferents.
- neurotransmission between primary afferents and dorsal horn neurons.

- somatotopically and "musculotopically" organized interneurons within the dorsal horn of the spinal cord, which can project either intraspinally or supraspinally.
- motor neurons and muscle groups that act synergistically to effect limb withdrawal.

Development of the cutaneous flexion withdrawal reflex

Reflex thresholds also demonstrate a developmental pattern. In both young rat pups and human infants, reflex withdrawal can be elicited by much weaker mechanical stimuli than those required in adults.(Fitzgerald M et al 1988). Up to P8, the reflex is sensitized by repeated stimulation and only after this time does it show the more mature pattern of habituation.(Fitzgerald M et al 1988) Thermal thresholds are also lower in neonatal pups than in adults.(Marsh DF et al 1999) Although sensitivity decreases with age, intensity discrimination and the range of behavioural responses develops over the first two weeks of neonatal life.(Collier AC et al 1980) This may possibly be due to either the generally less well developed organization of cutaneous nociceptive reflexes before the second postnatal week and/or the relatively weak intrinsic or descending inhibitory mechanisms in the rat spinal cord.(Jiang MC et al 1998; Fitzgerald M 1995)

As discussed in Chapter 1, during postnatal development, maturation of all stages of the nociceptive reflex occurs but changes in spinal cord connections are likely to be the most significant factor contributing to the development of reflex excitability. In early postnatal life, prior to the completion of C-fibre ingrowth and synaptic contact, large diameter Aβ myelinated fibres transducing low threshold stimuli make synaptic contacts within the SG of the superficial dorsal horn. In immature rats ESPCs can be elicited by Aβ afferents in SG neurons and innocuous inputs can lead to c-fos expression in the SG whereas in adults these are only possible with Aδ and C fibre afferents and noxious stimulation(Park JS et al 1999; Jennings E et al 1996) The receptive fields of the dorsal horn are also larger in the neonatal rat.(Fitzgerald M et al 1999) These large receptive fields and dominant A fibre input will increase the chance of central cells being excited by peripheral sensory stimulation and it is thought that this increased excitatory input maybe the basis of the exaggerated low threshold responses seen in rat pups and human infants.(Fitzgerald M 2000)

During these experiments the effects of opioid administration were measured in response to different stimuli. The cutaneous flexion withdrawal reflex gives a very clear reaction to a mechanical stimulus with a von Frey hair in animals at all ages including the young pups and was thus chosen as a model for testing the effects of the opioids.(Fitzgerald M et al 1988), though as already discussed non-noxious mechanical stimuli may elicit the reflex in younger animals. A thermal stimulus was used to investigate the effects of the opioids on C fibre stimulation, though again responses to C fibre stimulation are immature in the younger rat pups and activity of the withdrawal reflex following a thermal stimulus at this age will probably also involve stimulation of A fibres. Finally carrageenan inflammation was used as a model of tissue injury. Decreased thresholds following carrageenan occurs even in younger animals and this hypersensitivity provides a model for noxious stimulation at all of the ages.

2.1.4 Studies of mechanical sensory thresholds

Successive graded low intensity mechanical stimuli were applied to the dorsal surface of the hindpaw, using von Frey hairs (vFh), to measure the mechanical threshold of the cutaneous flexion withdrawal reflex. Von Frey hairs are single nylon monofilaments of graded diameter (0.08-1.0mm) attached at right angles to a Perspex handle, and are made according to a logarithmic scale of the weight that they apply. Each is calibrated using a balance accurate to 1mg, with a value being given to the weight produced on pressing the hair onto the balance, mimicking the action used during experimentation.

Table 1 shows the weights and natural logarithmic values of the numbered von Frey hairs when calibrated in our laboratory. The absolute values for their weights lie on an exponential scale, and since each of the age groups of the animals has a different baseline mechanical threshold, comparison of threshold changes between age groups is complicated. In order to allow comparison between the age groups, the natural logarithm of each weight is calculated and assigned a simple numerical value from 1 to 20, called the vFh number. This converts the thresholds to a linear scale and allows changes in thresholds to be compared even if the original baselines were not the same e.g. a change from vFh number 9 to 10 is equivalent to a change from vFh number 13 to 14. Using the natural logarithm the changes seen at any point on the scale are equivalent

and symmetric which is not the case when looking at percentage changes from the baseline. The percentage difference between two numbers is not symmetric and the result can change depending on which order the data is handled. (Cole T 2000)

A slightly different method of handling was required in each age of animal to allow testing: P3 animals were simply allowed to settle on the testing bench, P10 animals required minimal handling before testing to calm and reassure them whilst P21 animals required considerably more reassurance and comforting. Handling and testing was also much easier and at all age groups once the test opioid had been given, though there was no evidence of sedation at any of the ages or drug doses used, except in the initial experiments where P21 animals were given morphine at a dose of 4mg/kg. The ease of handling post opioid may be due to an anxiolytic effect of the opioid.

The von Frey hair producing the lowest force was pressed onto the dorsal surface, mid-paw of the left hind limb toe three times at one-second intervals. If this did not produce a flexion withdrawal reflex, then the process was repeated using the next von Frey hair. The threshold was obtained when a hair produced a flexion withdrawal of the limb, and the weight of that hair gave the threshold value. These initial thresholds were repeated after 5-10 minutes to check for reproducibility of the results.

During each experiment, a baseline mechanical threshold was obtained in all animals at time t_0. The opioid to be tested was then given by i.p. injection and the mechanical threshold again measured at times t_{15} (minutes), t_{30}, t_{45}, t_{60}, t_{75}, t_{90}, t_{120} and t_{150}. If the mechanical threshold had returned to approximately baseline at a time prior to t_{150} then recordings at all later time points were not necessarily made. During each batch of animals tested one, chosen at random, was injected with saline to act as a control. For each animal a response time curve could then be constructed.

Studies with adult animals

The maturation of the CNS in the rat is extremely accelerated compared with the human and is mature at 4 weeks.(Fitzgerald M et al 1993) Also by P40-50 O-demethylase activity in SD rats has declined to adult levels.(Matsubara T et al 1986)

Thus animals from these ages were regarded as adults. Female SD P63-70 (i.e. adult, mean weight 235.5g ±31.37) rats were obtained from the animal house at University College, London. All animals were kept fed and watered until the start of each experiment.

Experiments in this age group involved using von Frey hairs as a mechanical stimulus as described previously. Adult animals required more sustained handling than the younger rat pups and gentle restraint before testing could take place. Once more handling was easier once the test opioid had been given. Baseline mechanical thresholds were recorded from the left hind paw followed by i.p. injection of the opioid or saline to be studied, at a volume of 10ml/kg. Thresholds were then repeated every 15 minutes up to 90 minutes and again at 120 and 150 minutes.

Studies with the Dark Agouti strain

Female DA rats were purchased from Harlan OLAC, UK and were transported to the animal house at University College, London prior to each experiment with sufficient time to allow for acclimatization. All animals were kept fed and watered, and with their mother in the case of the rat pups, prior to the start of each experiment. Experiments involved comparisons of animals aged P3 (mean weight 8.86g ±0.54), P10 (mean weight 14.49g ±0.94), P21 (mean weight 44.45g ±4.3) and P63-70 (mean weight 147.83g ±6.31).

The DA rat is an inbred strain and is inherently small when compared to other strains of rat. Thus, the selection of rats from different strains, which are of similar body weights, would give groups of significantly different ages and thus they would not be matched in terms of development. Hence for experiments looking at the effects of development groups of rats were matched for age rather than weight.

Experiments with this strain of animal again involved using von Frey hairs as a mechanical stimulus. Adult animals required more sustained handling than the younger rat pups and gentle restraint before testing could take place and at each age group tested DA rats required more handling than SD rats, though again handling was easier once

the test opioid had been given. Baseline mechanical thresholds were recorded from the left hind paw followed by i.p. injection of the opioid or saline to be studied, at a volume of 10ml/kg. Thresholds were then repeated every 15 minutes up to 90 minutes and again at 120 and 150 minutes.

2.1.5 Studies with a thermal stimulus

Constant thermal stimulation was used to provide a selective C fibre mediated noxious stimulus. Initially limb withdrawal from a hot plate was tested but consistent results proved impossible to obtain from the small immature animals even under controlled conditions and it was felt that this technique did not allow sufficiently reliable testing of the analgesic efficacy of opioids.

Limb withdrawal from a hot water bath proved to be much more reliable. A pilot study was carried out to determine the optimum temperature of the water for each age group. This was the temperature that gave an easily recordable and reproducible baseline plus an increase from baseline following opioid administration that was long enough to show a significant change but not too long as to not produce the withdrawal. Again this varied between the age groups, being 45°C for P3, and 48°Cfor P10 and P21. Baseline withdrawal times were measured, using a stopwatch able to record one hundredth of second, by dipping the left hindpaw into the water and recording the time to withdrawal. This was repeated three times, with a five minute gap between each recording to allow the hind paw to dry and return to room temperature, with the average taken as the baseline withdrawal time. Opioid or saline was then injected via the intra-peritoneal route and repeat withdrawal times were then measured every 15 minutes up to one and a half hours and again at 2 hours. Only one recording could be taken on each occasion as it was not possible to allow time for the hind paw to dry and return to room temperature.

2.1.6 Carrageenan induced inflammation

Carrageenan induced inflammation was chosen as it confers a number of advantages over other inflammatory agents for the model we required:

- Subcutaneous carageenan injection results in a reliable unilateral and local inflammatory process.
- Carrageenan does not induce marked spontaneous behavioral signs of pain yet is known to reliably induce hyperalgesia to both mechanical and thermal stimuli.
- Subcutaneous carrageenan injection results in an inflammatory process with a convenient time frame i.e. onset within 2-3 hours and a persistence for up to 6 hours.
- Subcutaneous carageenan injection apparently does not induce a number of significant and unpleasant local and systemic side-effects as do some of the other chemicals used to produce an inflammatory model e.g. complete Freund's adjuvant.

Carrageenan is a mucopolysacharride derived from the Irish sea moss, Chondrus. The algae contains a mixture of at least two forms (κ and λ) of the polysacharride that each have molecular weights in excess of 100,000. The polymers consist of units of galactose and 3,6-anhydrogalactose which are esterified with sulphuric acid.(de-Lima J 2001)

Lambda carrageenan is prepared as a 1-2% non gelating hydrocolloid in saline and has been injected subcutaneously, intra-pluerally and intra-articularly to induce inflammation where it appears to produce only local inflammatory effects, is non-antigenic and is devoid of systemic side-effects.(Gardener DL 1960) Furthermore it does not appear to cause the release of either histamine or serotonin and shows good reproducibility with detectable regression in induced oedema in response to anti-inflammatory drugs.

Following injection of subcutaneous carrageenan in the rat, paw volume increases and peaks over 1-3 hours. This then stays constant for the next 24-96 hours before declining and is barely detectable at 7 days.(Bhattacharya SK et al 1987; Kayser V et al 1987)
The oedema is produced in two phases:(Vinegar R et al 1969)

1. An early transient phase in the first 20-60 minutes after injection that does not depend on the quality of the carrageenan injection and may be the result of the

trauma of the injection and /or due to a vasodilating co-compound or release of an autocoid such as serotonin from tissue mast cells.

2. A delayed phase due to the release and localisation of neutrophils, which are seen histologically after 20 minutes and have increased 10-fold by 60 minutes. A neutrophil chemotactic agent is generated and released which allows for this localisation. Neutrophils migrate into the extravascular space and cause phagocytosis of the carrageenan which results in lysosomal enzyme release and the activation of the prostoglandin biosynthetic pathway. A highly reactive intermediate species, as yet unidentified, is the likely candidate as the mediator of the capillary permeability changes that underlie the generation of oedema.(Vinegar R et al 1976) The oedema is directly related to the number of neutrophils that are mobilised and move into the extravascular space.(DiRosa M et al 1971) Recently NGF has also been shown to be a mediator involved in carrageenan induced inflammation.(Amann R et al 2000)

2.1.7 Studies with a mechanical stimulus plus inflammation

An inflammatory process was induced by the intraplantar injection of carrageenan. This has been shown as a reliable method of producing experimental inflammation in previous studies.(Hargreaves K et al 1988; Kayser V et al 1987) Prior to general anaesthesia, the mechanical thresholds, using von Frey hairs, were measured on the left hind paw and the contralateral (i.e. right) hind paw. General anaesthesia was then induced using a mixture of halothane 2-4% in 100% oxygen. Once anaesthesia was deep enough to prevent any response to stimulation, a subcutaneous injection of carrageenan 1% was made on the plantar surface of the left hind paw at a dose of 1μ ml/g in all age groups. Anaesthesia was then terminated and the animals returned to their mothers.

Initial control experiments involved animals from each age group receiving the carageenan injection with no further intervention. Mechanical thresholds were repeated on both hindpaws at hourly intervals up to three hours and then at 15-minute intervals up to 4 and a half hours and again at 5 hours and 6 hours. From these data it was found that the maximum drop in threshold occurred a 3 hours and that this stayed until 6 hours.

In subsequent experiments animals from each age group received the carrageenan injection followed by an i.p. injection of opioid or saline at 3 hours after the carageenan. Mechanical thresholds were repeated on both hind paws prior to the injection of the opioid or saline and then subsequently every 15 minutes for one and a half hours and also at 2 and 3 hours after the i.p. injection.

Studies with an opioid antagonist

Naloxone is a commonly used competitive antagonist at mu, delta and kappa opioid receptors.(Rang HP et al 1995) Neonatal Narcan, naloxone 20mcg/ml (University College Hospital Pharmacy) was diluted with sterile normal saline to give the concentrations required in a volume of 10ml/kg and was given via the i.p. route as described previously.

Baseline mechanical thresholds were recorded from both hind paws of each rat pup and then inflammation was induced in the left hind paw with carrageenan using the same techniques described. At 3 hours after the carageenan, mechanical thresholds were repeated. Two simultaneous i.p. injections were then given, naloxone plus the opioid or saline. Naloxone was given in large doses to attempt to achieve complete antagonism of the opioid effect i.e. one fifth of the dose of the opioid being given. Thresholds were then repeated in both hind paws every 15 minutes for the first one and a half hours and again at 2 hours and 3 hours. These experiments were performed on P3 and P10 animals using the following doses of drugs:
Morphine 2mg/kg + Naloxone 400mcg/kg
Codeine 10mg/kg + Naloxone 2mg/kg

Termination

At the conclusion of each experiment all the animals were sacraficed by the i.p. injection of Pentobarbitone (University College Hospital Pharmacy) in a dose suitable to produce terminal analgesia at each age group.

2.1.8 Analysis

Data from these experiments were analysed electronically using Microsoft Excel 2000 and Graphpad Prism 3.0. Comparisons between continuous variables were made using analysis of variance (ANOVA) with Bonferroni post test corrections. Categorical data was analysed using the chi-squared test. For all comparisons $p<0.05$ was considered significant.

vFh number	weight (gms)	$\log_e$
1	0.015	-4.12
2	0.04	-3.22
3	0.06	-2.81
4	0.12	-2.12
5	0.17	-1.77
6	0.36	-1.02
7	0.55	-0.59
8	0.92	-0.08
9	1.37	0.31
10	2.2	0.79
11	3.4	1.22
12	5.5	1.71
13	10.5	2.35
14	17.1	2.84
15	25.8	3.25
16	37	3.61
17	63.5	4.15
18	105	4.65
19	157	5.06
20	248	5.51

Table 1

Weights and $\log_e$ of each numbered von Frey hair when applied to a calibrated balance.

2.2 Results

The effects of codeine and morphine on the behavioural responses to mechanical stimulation were studied in SD and DA rats at different ages (i.e. P3, P10, P21 and P63-70). The effects of these drugs on the behavioural responses to thermal stimulation and mechanical stimulation following inflammation in SD rats in the younger age groups (P3, P10 and P21), were also studied.

In the experiments the end point recorded was the stimulus or latency to elicit the flexion withdrawal reflex in the left hindlimb. For mechanical stimulation this was done using the vFh index and for thermal stimulation this was the time to withdrawal recorded in seconds. In all cases this end point was compared to the baseline measure for each animal and the result was recorded as a change in vFh threshold or change in latency.

The number of animals studied in each group is with the results of the respective experiment. Unless otherwise stated, data was analysed using one and two way ANOVA with Bonferroni post-test corrections. A p value of <0.05 was considered significant.

2.2.1 Changes in baseline thresholds with age

Baseline vFh threshold

Figure 7 shows the mean baseline mechanical thresholds of the flexion withdrawal reflex in each age group for both SD and DA rats. These data are pooled from animals used in all the experiments.

The number of animals used at each age group was:

	SD (n)	DA (n)
P3	143	10
P10	140	27
P21	116	20
P63-70	8	6

The findings demonstrate the basic observation that in both strains the baseline mechanical threshold is lower in neonatal animals and increases with age up to adult values i.e. from 9.75 in the SD P3 animals to 17.5 in the SD P63-70 animals and from 8.4 in the DA P3 animals to 16.83 in the DA P63-70 animals. Analysis shows that there is an effect of both age and strain on the baseline threshold ($p<0.001$). Within both strains there is a significant difference between the baseline values at all age groups ($p<0.001$ in all cases). Comparison between the strains shows a significant difference between the baselines in the P3 (9.75 vs 8.4) and P10 (11.48 vs 9.22) age groups ($p<0.001$) but no difference at the P21 and P63-70 age groups.

Baseline thermal thresholds

An initial pilot study was done to find a suitable temperature for testing at each of the age groups. This was the temperature at each age that gave an easily recordable and reproducible baseline together with an easily measurable withdrawal time following morphine or codeine. This was found to be 45°C for the P3 pups and 48°C for the P10 and P21 pups. These temperatures were then used in the subsequent experiments.

Baseline thermal thresholds followed the same trend as the mechanical thresholds i.e. increasing with age. In each of the age groups n=10. For the P3 animals the mean baseline latency was 0.31s, for the P10 animals it was 0.45s and for the P21 animals it was 0.53s.

2.2.2 Effect of morphine and codeine on baseline mechanical thresholds

The change in mechanical threshold from baseline values over time was recorded in SD rat pups at ages P3, P10 and P21 following the i.p. injection of morphine 2mg/kg and a range of codeine doses from 7.5-30mg/kg. For each dose of opioid and each age group n = 10 animals. For analysis the data from each experiment was converted into two summary statistics:

1. Peak effect – this is the largest change in mechanical threshold from baseline recorded.

2. Area under the curve (AUC) – the change in mechanical threshold is recorded over time and the area under the curve is calculated.

Peak Effects

Figure 8 shows the mean peak changes in the baseline mechanical threshold. Comparison of all animals shows a significant interaction between age and drug and a significant effect of age and drug dose ($p<0.0001$).

Effect of morphine

Following morphine 2mg/kg there is a consistent rise in mechanical threshold in each of the age groups that is not significantly different between the age groups.

Effect of codeine

At all codeine doses, P3 animals show a much smaller response than P10 and P21 animals ($p<0.001$). At P3 the effect of codeine reaches a maximum at 10mg/kg, and is not increased by further increasing the dose to 15 or 30mg/kg.

For the P10 and P21 age groups at the lowest dose (7.5mg/kg) the response is greater in the P10 animals ($p<0.001$) but at higher doses the responses are similar and increase with dose.

Comparison of the effects of morphine and codeine

In contrast to codeine, morphine is equally effective at all 3 ages. P3 animals show a larger peak effect following morphine than at any of the doses of codeine ($p<0.001$). Morphine 2mg/kg and codeine 7.5, 10 and15mg/kg are equally effective at P10, and at P21 morphine 2mg/kg and codeine 10 and 15mg/kg are equally effective but at P21 codeine 7.5mg/kg is less effective than morphine 2mg/kg ($p<0.001$).

Time courses

Figure 9 shows the time courses for the mean change in the mechanical threshold. Comparison of all animals shows a significant interaction between age and drug and a significant effect of age and drug dose for morphine and codeine ($p<0.0001$).

Effect of morphine

Following morphine 2mg/kg the change in mechanical threshold with time is consistent in each of the age groups but the magnitude of this change is greatest at P10 ($p<0.05$). There is no difference for AUC between the P3 and P21 animals.

Effect of codeine

Once more the effect of codeine is less in the P3 animals and the maximum effect is reached at 10mg/kg, which is not increased by increasing the dose of codeine. In these P3 animals, the peak effect and duration of action are both smaller than in the P10 and P21 animals at all codeine doses ($p<0.001$). Also, in the P3 animals there is no difference in AUC between codeine doses of 10, 15 and 30mg/kg, but there is a smaller AUC at 7.5mg/kg ($p<0.001$).

Although there is no difference in the mean peak effect between P10 and P21 at codeine 10 and 15 mg/kg, the duration of action is longer in the P10 animals ($p<0.001$). At codeine 7.5mg, the peak effect and duration of action is greater in the P10 animals than the P21 animals ($p<0.001$).

Comparison of the effects of morphine and codeine

Again, in contrast to codeine, morphine is effective at all 3 ages. P3 animals show a much smaller response to codeine compared with morphine at all of the codeine doses ($p<0.001$).

At P10 there is no difference in response between morphine 2mg/kg and codeine 7.5mg/kg but there is a greater response to codeine 10 and 15mg/kg compared with

morphine 2mg/kg ($p<0.01$ and $p<0.001$ respectively). However, at P21 the pattern of response is different, there is a greater response to morphine 2mg/kg compared with codeine 7.5mg/kg ($p<0.001$), no difference in response between morphine 2mg/kg and codeine 10 mg/kg and a greater response to codeine 15mg/kg in comparison with morphine 2mg/kg ($p<0.01$).

Effect of saline

From each litter tested one animal was chosen at random to receive i.p. saline instead of the opioid. In all these animals, irrespective of age, the mechanical threshold did not change from baseline at any time point.

Rationalisation of drug doses

From these initial experiments, using a mechanical stimulus, it was hoped to identify comparable doses of codeine and morphine that could be used in subsequent experiments. However, due to the differences in effects across the age groups in was not possible to identify exactly equipotent doses applicable at all of the ages. Codeine 10mg/kg and morphine 2mg/kg gave the closest approximation to equipotent doses and were thus used for comparison in the further behavioural studies.

SD adult animals

Because of the age related differences in activity of SD rat cytochrome P450 it was decided to repeat the experiments in adult rats for a comparison with the younger age groups. Figure 10 shows the time course for the mean change in mechanical threshold adult SD rats following i.p. codeine 10mg/kg and morphine 2mg/kg ($n = 4$).

From figure 7 it can be seen that the mean baseline mechanical threshold in this age group is 17 – 18. This is close to the top end of the available vFh range, which ends at a vFh number of 20. Thus, following the injection of the opioid to be tested, at some time points the threshold was not reached when the strongest von Frey hair was used. For the

purposes of graphical representation in figure 10, where all the animals did not respond to the strongest stimulus at a certain time point an arbitary data point is shown in the upper portion of the graph, which is divided by a segmented Y-axis, and at time points where some animals responded whilst others did not, those not responding are given an arbitary vFh index of 21 to allow for a data point to be shown.

Since it was not possible to record precise data points statistical comparisons between the two drugs tested and with the other age groups are not possible. However, in the cases of both codeine and morphine, there is a consistent and significant increase in mean mechanical threshold following i.p. injection.

Experiments with DA animals

The DA rat is thought to lack the enzyme responsible for the metabolism of codeine to morphine and has been used as a model of a PM of codeine.

Adult DA animals

Adult DA animals show a smaller response to codeine compared with adult DA rats treated with morphine and adult SD rats treated with either codeine and morphine. Figure 10 shows the time course for the mean change in mechanical threshold of adult DA rats following i.p. codeine 10mg/kg and morphine 2mg/kg ($n = 3$). From figure 7 it can be seen, as with the SD rats, that the baseline mechanical threshold is near to the top end of the vFh range. Thus, for graphical purposes in figure 10, the same descriptive methods are applied as described for the adult SD rats in the previous section.

A comparison was made between DA rats treated with codeine and SD treated with codeine and DA rats treated with morphine. In each case the data were analysed using a chi-square test, taking the 45-minute time point and comparing the proportion of animals in each group with thresholds that were higher than that elicited by the von Frey hair of the largest index. There is a significant difference (between the change in mechanical threshold) between adult DA rats treated with codeine and morphine and also between adult SD and DA rats treated with codeine, ($p<0.001$). However, due to

being unable to record precise data points when the reflex could not be elicited by the strongest vFh comparison of the effects of morphine between the two adult strains and comparisons with other age groups were not possible.

DA rat pups

Figure 11 shows the mean peak changes in the baseline mechanical threshold of DA rat pups at ages P3, P10 and P21 following i.p. morphine 2mg/kg and codeine 10mg/kg. For each dose of opioid and each age group n = 5 animals. The results following both opioids were not quantitatively different from those seen in the SD animals at all ages.

Effect of morphine

Following morphine there is a consistent rise in mechanical threshold in each of the age groups that is not statistically different between the age groups..

Effect of codeine

As with the SD rats, DA P3 pups also show a smaller increase in the mean peak mechanical threshold compared with both the P10 and P21 DA pups ($p<0.001$). Similarly, in the DA P10 and P21 age groups following codeine there is again a consistent rise in the mean peak mechanical threshold that is not different between the two groups.

Comparison of the effects of morphine vs codeine

Comparing morphine and codeine across the age groups in the DA pups shows that in the P3 animals there is a smaller effect in the codeine animals ($p<0.001$). Whilst in the P10 and P21 animals there is no difference in effect between morphine and codeine.

2.2.3 Effect of morphine and codeine on baseline thermal latencies

Figure 12 shows a comparison of the change in the time from baseline to elicit the flexion withdrawal reflex on immersion in hot water following the i.p. injection of codeine 10mg/kg, morphine 2mg/kg and saline in SD rat pups (n = 5 for the opioid animals and n=1 for the saline animals).

These data were analysed by taking the maximum change from baseline, in seconds, in time taken to elicit the flexion withdrawal reflex after intra-peritoneal injection of the drug. Comparison of all animals shows a significant interaction between age and drug with $p=0.0071$ and a significant effect of age with $p=0.0019$ but with no significant effect of drug.

Effect of morphine

Morphine consistently increased the latency with no relative differences across the age groups.

Effect of codeine

Codeine is much less effective at increasing the latency in the P3 animals. P3 animals given codeine show a much shorter latency compared to the P10 and P21 pups ($p<0.001$). There is no difference in latency in the P10 and P21 animals following codeine.

Comparison of the effects of morphine and codeine

In contrast to codeine, morphine is equally effective at all 3 ages. At P3 there is a smaller effect in the animals treated with codeine compared with those animals treated with morphine ($p<0.01$). Whilst in the P10 and P21 animals there is no significant difference (in effect) between morphine and codeine.

Effect of saline

From each litter tested, one pup was selected at random and given an i.p. injection of saline instead of the opioid. The latency in these animals in all the age groups did not differ from baseline throughout the experiments. There was also no difference in the P3 animals between those treated with saline and those treated with codeine.

2.2.4 Effect of carrageenan on mechanical thresholds

An inflammatory reaction was produced in the hindpaw of SD rat pups. Following determination of the baseline mechanical threshold the rat pups were given a brief general anaesthetic of halothane in 100% oxygen, during which time they were given an injection of carrageenan 1μml/g into the plantar surface of the left hindpaw. The change in mechanical threshold was then measured over time.

Control animals

After the injection of carrageenan, in all age groups (n = 4) the threshold decreased with time to a maximum at 3 hours, with this decrease remaining constant up to 6 hours when the measurements were terminated,representing a hypersensitivity to mechanical stimulation. Further experiments were then performed comparing the effects of opioid or saline using 3 hours post carrageenan injection as the time of injection of the study drug

Effect of age on carrageenan induced hypersensitivity

Figure 13 shows a comparison of the mean change in the mechanical threshold in the left (ipsilateral) and right (contralateral) hindpaws of SD rat pups 3 hours following the injection of carrageenan into the plantar surface of the left hindpaw. These data are taken from all the animals injected with carrageenan during the experiments for this thesis (P3 n = 31, P10 n = 31 and P21 n= 20).

Effect on ipsilateral paw

In the ipsilateral paw the maximum reduction in threshold after carrageenan increases with increasing age of the animal. There is a difference between the P3 and both the P10 and P21 pups ($p<0.001$) though there is no significant difference between the P10 and P21 pups.

Effect on the contralateral paw

Decreases in threshold in the contralateral paw were also observed but this not differ between the age groups or from the baseline in the P21 animals. However, there is a decrease in threshold compared with the baseline for the P3 and P10 pups ($p<0.01$ and $p<0.001$ respectively).

2.2.5 Effect of morphine and codeine on carrageenan induced hypersensitivity

Ipsilateral paw

Figure 14 shows the effects of i.p. opioid or saline on carrageenan induced hypersensitivity in P3, P10 and P21 SD rat pups. The animals in each of the opioid groups ($n = 6$) received an i.p. injection of either codeine 10mg/kg or morphine 2mg/kg. These data were analysed by taking the maximum change in mechanical threshold in comparison with the carrageenan reduced threshold for each individual animal. Comparison of all animals shows a significant interaction between age and drug and a significant effect of age and drug dose ($p<0.0001$).

Effect of morphine

Morphine reduces the hypersensitivity due to carrageenan at all ages. The magnitude of this effect varies across the age groups being greater in the P10 and P21 pups. Following injection of morphine the mechanical threshold is increased above baseline. This response diminishes with time and the mechanical threshold returns to the level 3

hours post injection of carrageenan in all animals. There is no difference in the maximum effect of morphine between the P10 and P21 pups but there is a smaller maximum effect of morphine when comparing the P3 animals with the P10 and P21 animals ($p<0.001$ in both cases).

Effect of codeine

Once again codeine is much less effective in the P3 animals compared with the older animals. In the P3 pups carrageenan hypersensitivity is not reversed. There is no difference in effect between P3 animals treated with codeine and the P3 saline and naïve controls.

As with animals given morphine, both the P10 and 21 animals given codeine increase the mechanical threshold back above the baseline. As the effect of the opioid diminishes with time the mechanical threshold returns back to the level 3 hours post injection of carrageenan. The maximum effect of codeine is not different at either of these ages.

Comparison of the effects of morphine and codeine

Once again morphine is effective at all 3 ages whereas codeine is not. In the P3 animals there is no effect following the injection of codeine compared with a significant effect following morphine. However, there is no difference between the maximum effects seen in the P10 and P21 animals receiving codeine and morphine.

Effect of saline

The animals receiving saline after carrageenan ($n = 4$), in all age groups, showed a decrease in baseline to a maximum at 3hrs, which then remained constant to the end of the experiment at 6 hours. There was no difference between these animals at any of the age groups.

Contralateral paw

Figure 15 shows the effect on the mean vFh threshold of the contralateral hindpaw in the same experiments on the SD rat pups injected with carrageenan described above. Once again these data were analysed by taking the maximum change in mechanical threshold in comparison with the carrageenan reduced threshold for each individual animal. Comparison of all animals shows a significant interaction between age and drug and a significant effect of age and drug dose, all with $p<0.0001$.

Effect of morphine

Once more the effect of morphine is similar at each age group but the magnitude of this effect varies across the age groups. Following injection of morphine the mechanical threshold is increased above baseline. This response diminishes with time and the mechanical threshold returns to the level 3 hours post injection of carrageenan in all animals. There is no difference in the maximum effect of morphine between the P3 and P21 animals and between the P10 and P21 animals but there is a greater effect seen in the P10 animals compared with the P3 animals ($p<0.01$).

Effect of codeine

As with the ipsilateral paw codeine is much less effective in the P3 animals compared with the older animals. In the P3 pups carrageenan hypersensitivity is not reversed. There is no difference in effect between P3 animals treated with codeine and the P3 saline and naïve controls.

As with animals given morphine, both the P10 and 21 animals given codeine increase the mechanical threshold back above the baseline. As the effect of the opioid diminishes with time the mechanical threshold returns back to the level 3 hours post injection of carrageenan. The maximum effect of codeine is not different at either of these ages.

Comparison of the effects of morphine and codeine

The pattern of the results of previous experiments is repeated where morphine is effective at all 3 ages but codeine is not. In the P3 animals there is no effect following the injection of codeine compared with a significant effect following morphine. However, there is no difference between the maximum effects seen in the P10 and P21 animals receiving codeine and morphine.

Effect of saline

The animals injected with i.p. saline show a small but not significant decrease in mechanical threshold from baseline that remains constant throughout the time of the experiment. There was no significant difference between these animals at any of the age groups.

2.2.6 Effect of adding naloxone

The experiments involving the use of carrageenan followed by subsequent injection of opioid were repeated in P3 and P10 SD rat pups (n = 5) using exactly the same protocol as the previous experiments. However, on this occasion, at 3 hours an intra-peritoneal injection of the mu receptor antagonist, naloxone, was given in addition to the opioid to be studied. The dose of naloxone used was one-fifth of that of the opioid given i.e. 400mcg/kg with morphine 2mg/kg and 2mg/kg with codeine 10mg/kg.

Figure 16 shows the mean changes in mechanical threshold of the ipsilateral hindpaw in P3 and P10 SD rat pups given simultaneous injections of naloxone and opioid 3 hours after intra-plantar injection of carrageenan. The graphs also show the results of the experiments using opioid alone post carrageenan in these age groups for comparison.

These data were analysed by taking the maximum change in mechanical threshold in comparison with the carrageenan reduced threshold for each individual animal. Comparison of all animals shows a significant interaction between age and drug and a significant effect of age and drug dose, all with $p<0.0001$.

Effect of naloxone on morphine analgesia

Following morphine and naloxone there is initially no change in the mean mechanical threshold. At 60 –75 minutes after the opioid and naloxone injection, in both age groups, the mechanical threshold begins to increase and the decreased threshold is reversed, increasing marginally above the baseline before returning to the level at 3 hours after carrageenan injection by the end of the experiment. In both age groups the overall effect of the opioid is less than in those animals given the opioid alone ($p<0.001$ in each case). As with the experiment without naloxone, there is also a greater effect in the P10 animals compared with the P3 animals ($p<0.001$).

Effect of naloxone on codeine analgesia

The effect of adding naloxone to codeine is different when comparing the two age groups ($p<0.001$). There is no difference between the P3 pups injected with codeine with or without naloxone. However, the P10 pups treated with codeine and naloxone initially show no increase in mean vFh threshold following injection of codeine but at around 60 minutes the threshold begins to increase and rises marginally above baseline before returning to the level at 3 hours after carrageenan injection by the end of the experiment. Again there was a difference in the overall opioid effect between these P10 pups and those given codeine alone ($p<0.01$).

Effect of naloxone, comparison of morphine and codeine

Naloxone initially prevented the rise in threshold following both codeine and morphine. Once more there is a significantly greater maximum effect produced in the P3 pups injected with morphine and naloxone than in those injected with codeine and naloxone ($p<0.001$), but in the P10 animals there is no difference in maximum effect between the animals treated with codeine or morphine plus naloxone.

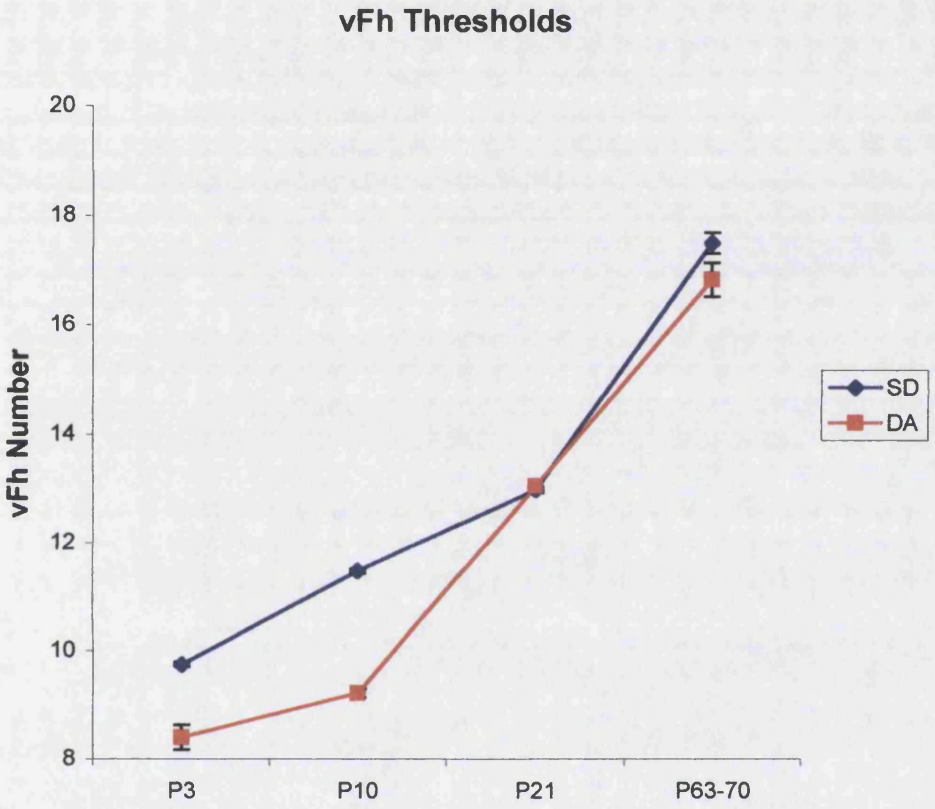

Figure 7. A comparison of the effect of age on the mean baseline von Frey hair thresholds in SD and DA rats. Displayed as means and standard errors of the mean.

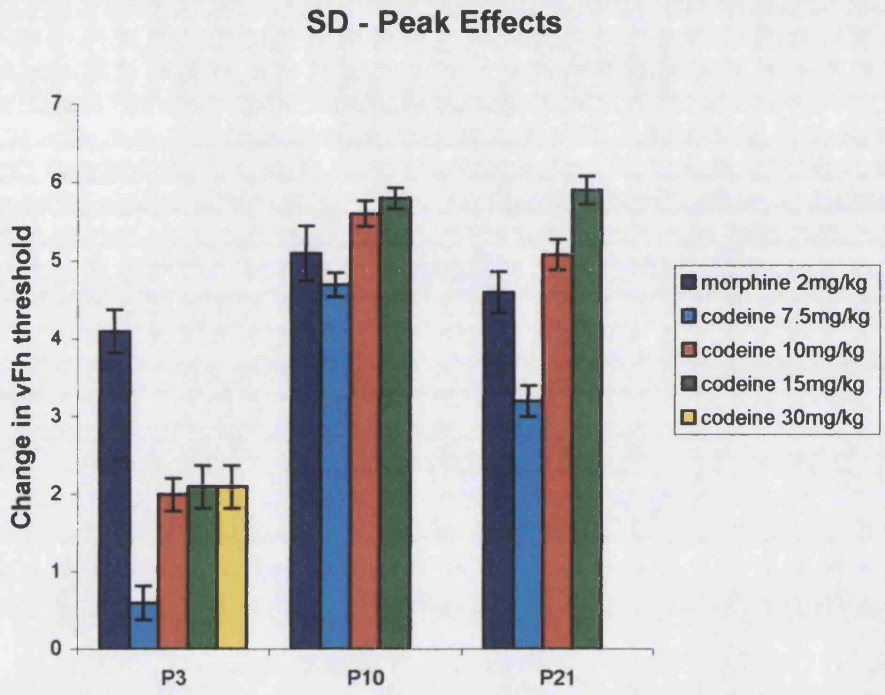

Figure 8. A comparison of the peak change in the vFh threshold following the administration of intra-peritoneal codeine or morphine to SD rat pups. Displayed are means plus standard errors of the mean. ANOVA was used to analyse these data (see text).

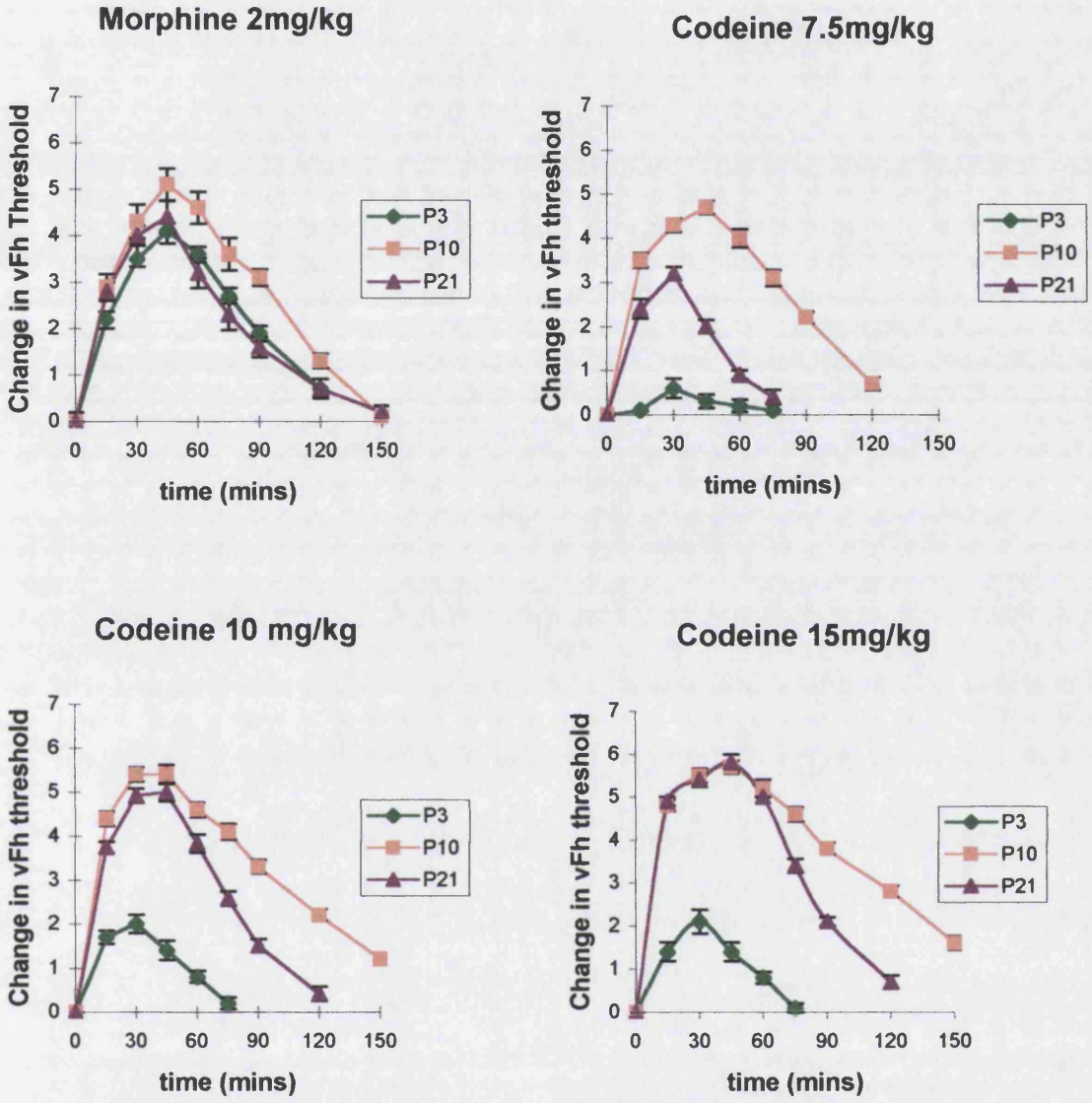

Figure 9. A comparison of time courses for the mean change from baseline in the vFh threshold in SD rat pups following intra-peritoneal injection of opioid. Displayed are mean and standard error of the mean. ANOVA was used to analyse these data (see text).

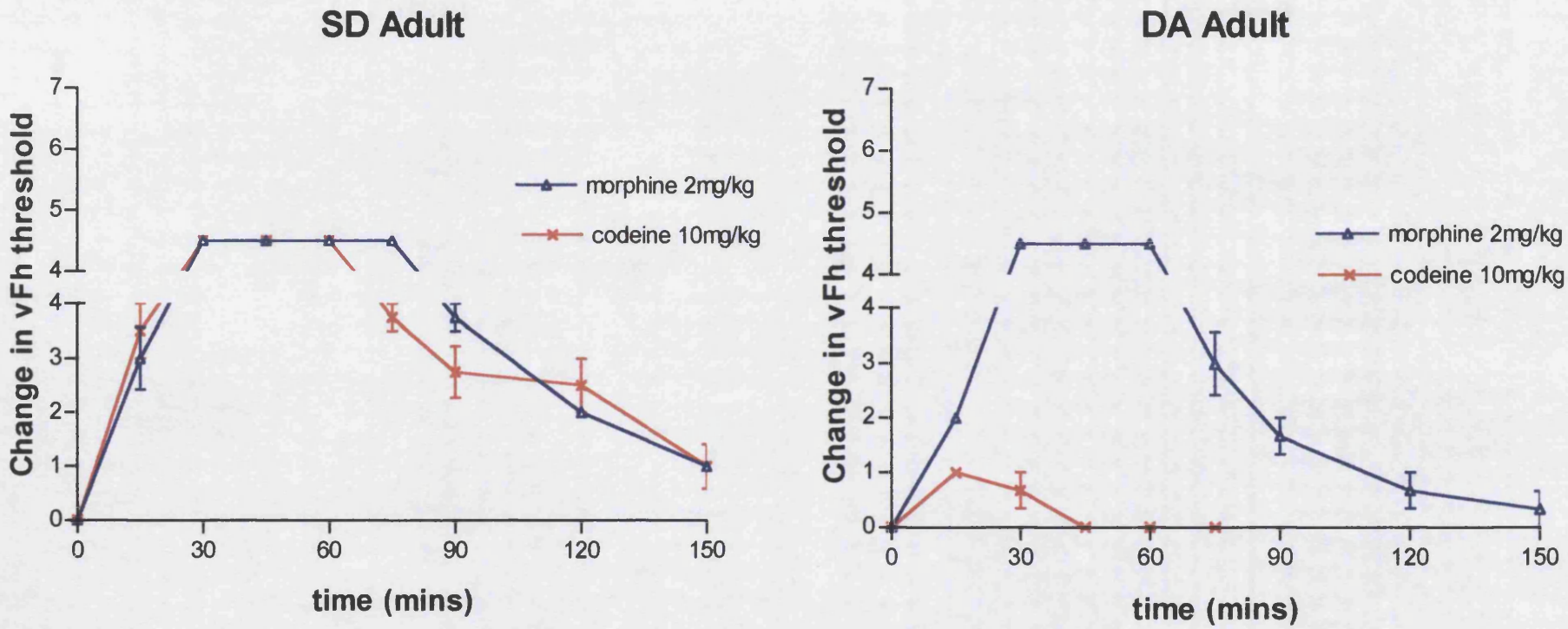

Figure 10. Comparison of the response versus time curves for the change in vFh threshold in adult SD and DA rats following intra-peritoneal injection of codeine and morphine. Displayed are means and standard error of the mean. The Y-axis is segmented. Values above the cut-off are arbitary as none of the animals at these time points responded to the von Frey hair with the highest index (see text).

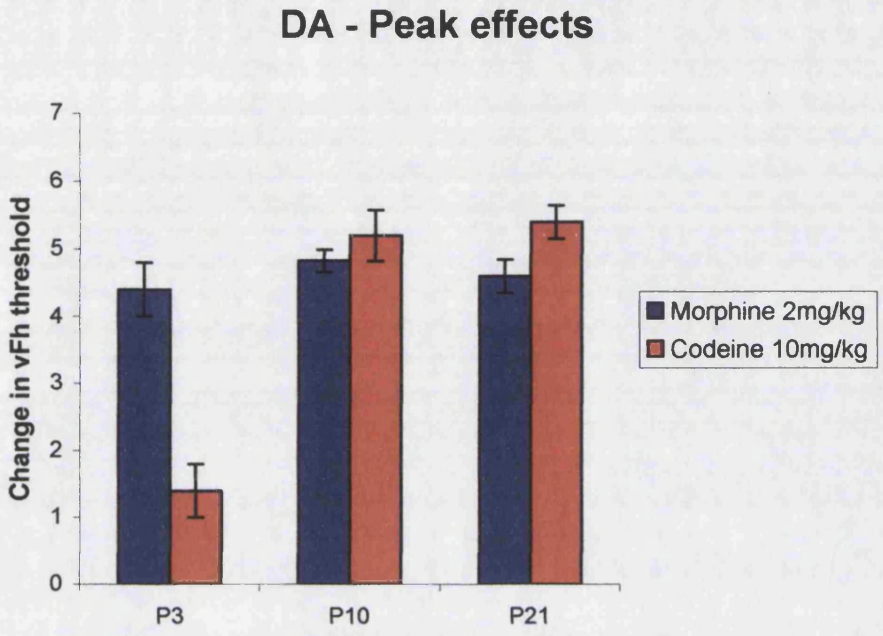

Figure 11. A comparison of the peak change in the vFh threshold following the administration of intra-peritoneal codeine or morphine to DA rat pups. Displayed are means and standard errors of the means. ANOVA was used to analyse these data (see text).

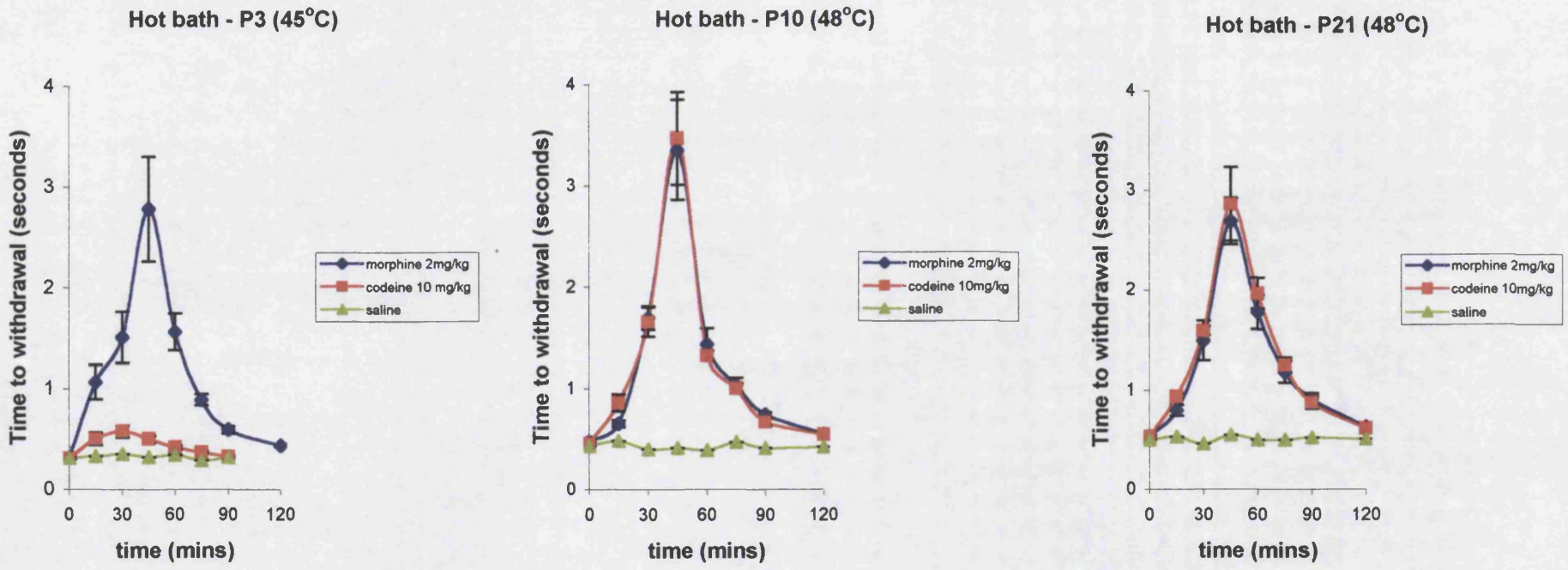

Figure 12. A comparison of the change in the time to withdrawal of the left hindpaw on immersion in hot water following intra-peritoneal injection of codeine, morphine or saline in SD rat pups. Displayed are mean and standard error of the mean. ANOVA was used to analyse these data (see text).

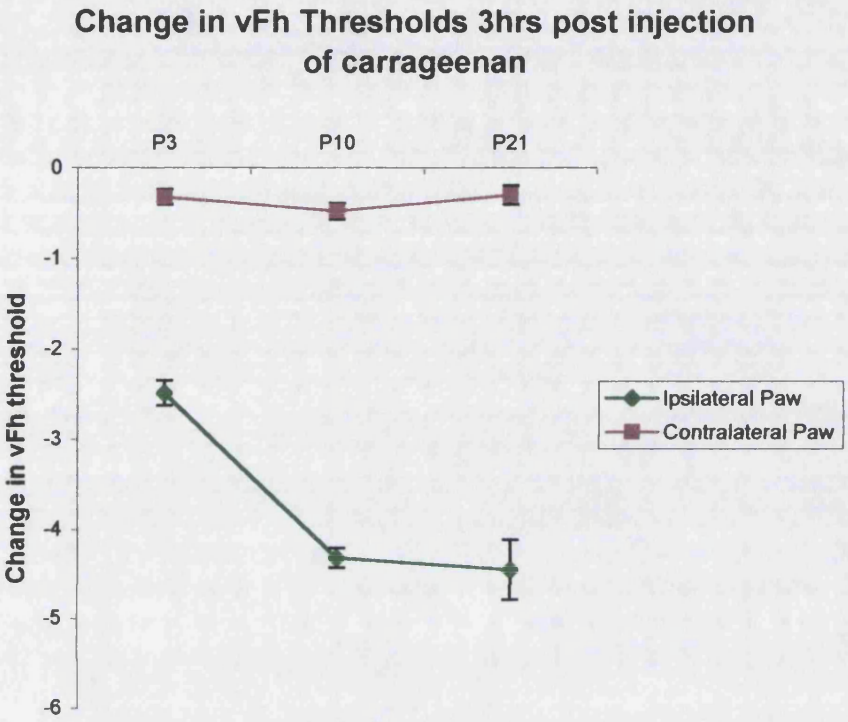

Figure 13. A comparison of the change in vFh threshold in the ipsilateral and contralateral hindpaws of SD rat pups 3 hours following the intraplantar injection of carrageenan into the left hindpaw. Means and standard errors of the mean displayed. ANOVA was used to analyse these data (see text).

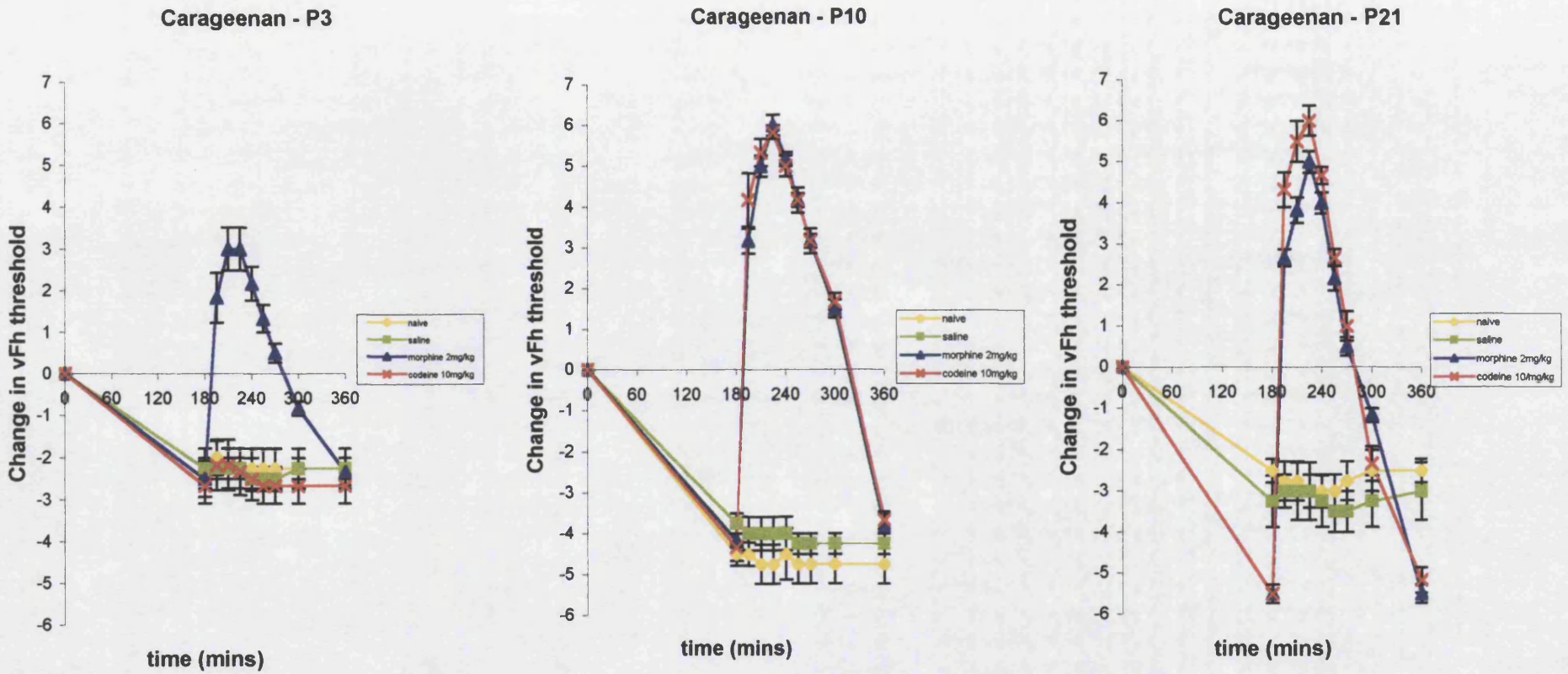

Figure 14. A comparison of the change in vFh threshold in the ipsilateral hindpaw following intra-peritoneal injection of codeine, morphine or saline in SD rat pups with left hindpaw inflammation post injection of carrageenan. Injection of carageenan performed at 0 mins followed by injection of opioid or saline at 180 mins. Mean and standard error of the mean displayed.

ANOVA was used to analyse these data (see text).

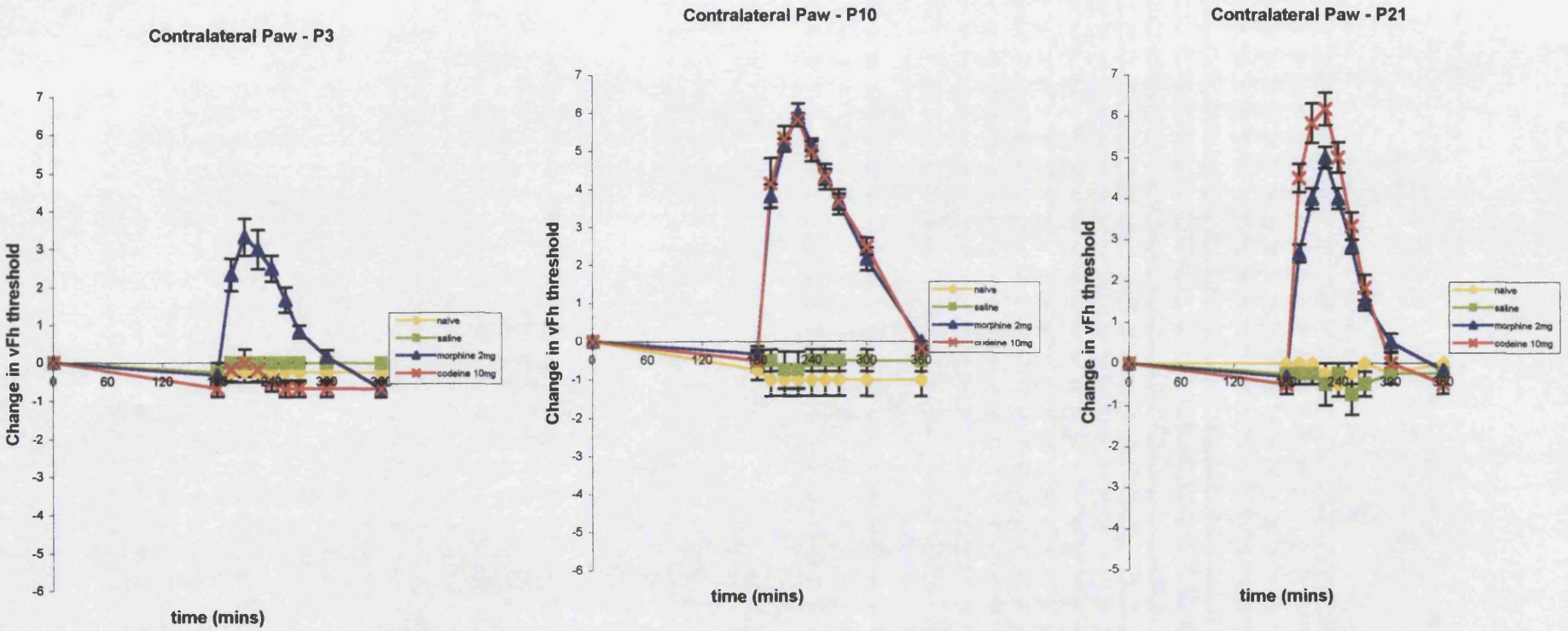

Figure 15. A comparison of the change in vFh threshold in the contralateral hindpaw following intra-peritoneal injection of codeine, morphine or saline in SD rat pups with left hindpaw inflammation post injection of carrageenan. Injection of carageenan performed at 0 mins followed by injection of opioid or saline at 180 mins. Displayed are means and standard errors of the mean. ANOVA was used to analyse these data (see text).

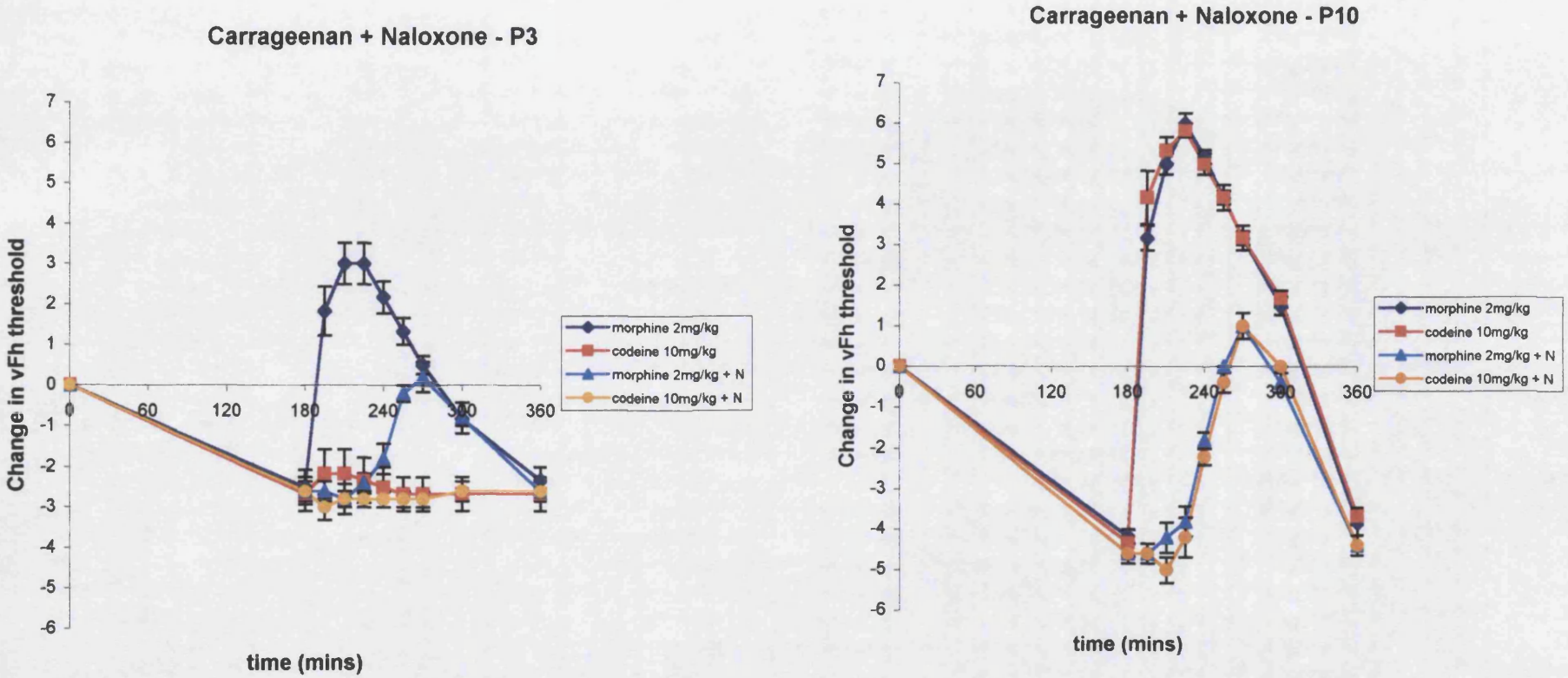

Figure 16. A comparison of the change in vFh threshold in the ipsilateral hindpaw following intra-peritoneal injection of codeine or morphine with or without simultaneous injection of naloxone in P3 and P10SD rat pups with left hindpaw inflammation post injection of carrageenan. Injection of carageenan performed at 0 mins followed by simultaneous injection of opioid and naloxone or opioid alone at 180 mins. N = naloxone at one-fifth of the dose of the opioid used. Displayed are mean and standard error of the mean. ANOVA was used to analyse these data (see text).

2.3 Discussion

The effects of morphine and codeine have been investigated in the rat during development using three models of nociceptive processing. Interspecies differences in the effects of codeine and morphine on mechanical sensory thresholds were also studied. The results from both SD and DA rat strains show there is developmental regulation of the efficacy of codeine. Further studies investigated the opioid receptor mediated mechanism of codeine analgesia in SD rats using the opioid antagonist naloxone and showed that codeine analgesia is mediated by an opioid effect.

2.3.1 Developmental changes in baseline thresholds

Mechanical

The cutaneous flexion withdrawal reflex in adult rats is well established as a tool in pain research, and has been extensively used in assessment of antinociceptive agents.(Woolf CJ 2001; Woolf CJ et al 1985) The current experiments confirm that in young rat pups, as in human infants, the flexion withdrawal reflex can be elicited by a weaker mechanical stimulus than that required in adults and that the force of the mechanical stimulus required to elicit the reflex increases with age.(Andrews KA et al 1994; Fitzgerald M et al 1988)

Changes in spinal cord connections are considered to be the most important factor affecting the development of reflex thresholds. In the adult the reflex is mediated by Aδ and C fibres which both synapse in the superficial areas of the dorsal horn. However, in early postnatal life, prior to the completion of C-fibre ingrowth and synaptic contact, A β fibres, which are able to transduce low threshold stimuli, also synapse within the superficial dorsal horn. In immature rats ESPCs can be elicited by Aβ afferents in SG neurons and innocuous inputs can lead to c-fos expression in the SG whereas in adults these are only possible with Aδ and C fibre afferents and noxious stimulation(Park JS et al 1999; Jennings E et al 1996) The receptive fields of the dorsal horn are also larger in the neonatal rat.(Fitzgerald M et al 1999) These large receptive fields and dominant A fibre input will increase the chance of central cells being excited by peripheral sensory

stimulation and it is thought that these factors plus increased excitability of the dorsal horn neurons, due to a lack of descending and local inhibition in the first three post natal weeks is responsible for the exaggerated, low threshold responses seen in rat pups and human infants.(Fitzgerald M 2000; Jiang MC et al 1998; Coggershall RE et al 1996; Fitzgerald M 1995)

At P3 and P10 the DA animals have lower thresholds than the SD animals though in the older animals there is no difference. DA rats are an inbred strain and are smaller physically at all age groups than the SD animals. These differences in threshold in the younger animals may reflect different rates of neurological development between the strains, such as potentially different timings of synaptic changes or descending and inhibitory control.

Thermal

The response to noxious heat, such as the tail flick test, hot plate and hot water bath, has also been frequently used in pain research and in assessment of antinociceptive agents. Thermal stimuli are mainly mediated by C fibres and are thus considered a good model for nociception. Following thermal stimuli the same basic trend in response is seen, with the baselines increasing with age. The time to limb withdrawal was shortest at P3 even though a lower temperature was also used at this age. These results are consistent with previous studies, which have shown lower temperatures are required and withdrawal times are shorter in younger animals.(Marsh DF et al 1999; Conway CM et al 1998)

A number of factors could account for these differences: developmental changes in skin thickness and texture, differences in the amount of subcutaneous fat, relatively deeper positioning of thermal nociceptors in older animals or differences in afferent fibre function and their central connections.(Conway CM et al 1998) In the adult rat most noxious heat information is transmitted by C fibres but a small proportion is carried by Aδ fibres.(Lynn B 2001) In early postnatal life C fibre connections are immature and responses maybe more reliant on mediation by Aδ fibres. The lower thresholds are most probably due to the same alterations in afferent fibre synapses in the dorsal horn, with

an involvement of Aβ fibres, and the increased level of excitability of the neonatal spinal cord previously mentioned.

2.3.2 Effect of morphine and codeine on baseline thresholds

The current experiments, using both mechanical and thermal stimuli have shown there is substantial developmental regulation of the efficacy of codeine, with the younger animals being less sensitive. In contrast the effects of morphine were broadly similar across the age groups studied. Comparison of SD and DA rats revealed both interspecies and developmental differences in codeine efficacy.

Adult studies

SD rats are known to be able to convert codeine to morphine and are used as a model of an EM of substrates that require CYP2D6 for metabolism whereas female DA rats lack CYP2D1, the supposed equivalent of human CYP2D6, and are used as a model of a PM.(Matsunaga E et al 1989; Boobis AR et al 1986) In the current experiments, adult SD and DA rats following morphine and adult SD rats following codeine all showed increases in baseline threshold to a mechanical stimulus. The magnitude of these responses could not be compared between these groups or with the younger animals as following administration of the opioid, since a response was not seen even with the largest vFh hair at some time points.

In adult DA animals treated with codeine, the response seen was significantly smaller than that of the other adult groups. Previous studies using female adult DA rats have also shown decreased morphine production in liver microsomes in vitro and significantly decreased analgesic efficacy following codeine administration in comparison with SD rats.(Cleary J et al 1994; Mikus G et al 1991) Thus, the results of the current experiments are in agreement with the previous studies and with consistent withthe theory that it is the metabolism to morphine that is wholly or partly responsible for the analgesic effect of codeine.

Studies with SD pups

Morphine

Morphine increases mechanical sensory thresholds to a similar extent at all ages tested. However, the AUC was greater for P10 animals, suggesting that morphine may have a longer duration of action at this age. Morphine also consistently increases the time to withdrawal following a thermal stimulus, again with no difference in the magnitude of the effect between the P3, P10 and P21 animals.

Conflicting results have been shown in previous behavioural studies of the effects of morphine in the developing animal, though different experimental designs were used. Following a thermal stimulus, morphine efficacy increased from P3 to P14 whilst using the tail flick test, no detectable effect was found until P12.(Giordano J et al 1987; Barr G et al 1986) In contrast other studies have shown the effects of morphine to be greater in younger animals. One study showed the peak analgesic effect was at P1 to a thermal stimulus whilst more recent work using the flexion withdrawal reflex found efficacy greatest at P3 and lowest at P21.(Marsh DF 1997; Blass EM et al 1993) Electrophysiological study has shown the effect of morphine is greater at P14 and P21 than in the adult rat.(Rahman W et al 1998)

In adults the effects of morphine are known to be primarily C fibre mediated and the majority of opioid receptors are located around C fibre terminals in the superficial dorsal horn.(Dickenson AH 1994) There is now evidence that mu receptors may also be located on A fibres in the neonate and are downregulated during development.(Beland B et al 2001) Thus morphine may act in the younger animals by blocking the Aβ fibre component of the reflex. A sedative effect has been suggested as an explanation for the response to opioids seen in the younger animals, however this has been shown to be quantitatively and qualitatively different from an analgesic effect in neonatal rats.(Abbott FV et al 1995)

Codeine

In the current experiments the effects of codeine on mechanical and thermal thresholds show consistent developmental regulation with the younger animals being less sensitive. In P10 and P21 animals, codeine increases the mechanical threshold in a dose related manner. In terms of maximum change in threshold from baseline there is no difference between the two age groups apart from at the lowest dose (7.5mg/kg), where the maximal effect is at P10. Though, at all doses of codeine the duration of action is longer in the P10 animals than the P21 animals.

In the P3 animals there is a markedly smaller response seen to codeine, at all doses the maximum effect and duration of action are significantly less than that seen at both P10 and P21. In addition, the overall response to codeine is maximal at 10mg/kg and cannot be increased even at doses of 30mg/kg i.e. a ceiling effect.

The same pattern of results were seen in response to a thermal stimulus following codeine, though in these experiments only one dose of codeine was used. Codeine was much less effective at increasing the latency in the P3 animals than in the older animals but between the older animals there was no difference in effect.

Following both mechanical and thermal stimuli at P10 and P21, the magnitude of response with the two opioids is broadly similar. However, at P3 the response to morphine is much greater than that to codeine at all codeine doses.

Previous animal and human experimental studies suggest that the conversion of codeine to morphine is wholly or mostly responsible for the analgesic efficacy of codeine. SD rats are known to be able to convert codeine to morphine and thus it can be expected that morphine will play a significant role in the effect of codeine seen in the current experiments.

One potential explanation of the decreased effect seen in the P3 animals following codeine, is that low activity of the enzyme converting codeine to morphine results in decreased effects. In the P10 and P21 animals O-demethylase activity is high and would be expected to allow the conversion of codeine to morphine in significant

amounts.(Matsubara T et al 1986) However, at P3, total enzyme activity is known to be about one-fifth of the activity at P10 and P21.(Matsubara T et al 1986) Thus the amount of morphine produced from codeine would be expected to be reduced, thus resulting in the decreased effects. This may also explain the inability to increase the effect of codeine when using doses greater than 10mg/kg. The decreased enzyme activity would potentially only allow a limited metabolism of codeine to morphine, which cannot be increased by increasing the amount of substrate.

Another possible explanation is that either codeine itself, or another metabolite has a direct analgesic action and that there is further developmental regulation of this effect. However, this is unlikely, as this would assume only a minor role for morphine in the analgesic effect of codeine, which in conflict with previous studies.(Eckhardt K et al 1997; Cleary J et al 1994; Sindrup SH et al 1992; Desmeules J et al 1991; Sindrup SH et al 1990)

Studies with DA pups

The studies involving DA animals at P3, P10 and P21 produced surprising results in that the responses to mechanical stimuli following both codeine and morphine exactly mirrored those seen in SD animals at the same ages. Clearly there is also developmental regulation of the efficacy of codeine in DA rats, which has important implications for its use as an animal model of a PM of CYP2D6 as enzyme activity may vary with age.

A possible explanation is that these younger DA animals are able to produce morphine from codeine. The role of the DA rat as a model for human PMs has been questioned, as other factors such as age and substrate concentration have are known to affect CYP2D1 efficiency at different ages in the adult. Also the handling of some compounds in rats may be different compared with humans and involve more than one cytochrome P450.(Barham HM et al 1994; Vincent-Viry M et al 1988; Boobis AR et al 1986; Khan GC et al 1985) CYP2D1 is not the only CYP2D isosyme present in the rat, CYP2D2 - 5 have also been identified.(Kawashima H et al 1995; Matsunaga E et al 1990; Gonzalez FJ et al 1987) The role these other enzymes may play in codeine metabolism is unknown, as is their relative contribution during development and between the two

strains. An explanation for the different effects of codeine seen during development and between the strains maybe that the activity of different enzymes changes during development and also between strains.

Unlike in SD rats, O-demethylase activity in DA rats has not been investigated. (Matsubara T et al 1986) If a similar pattern is present in DA animals then similar results may be expected with low activity at P3 rising to a peak at P10 and P21. The activity would then fall away to adult levels. Thus another possible explanation for the lack of effect in adult DA animals is that the activity of the CYP2D1 enzyme in the adult animals has fallen to low levels and the metabolism to morphine is not great enough to produce an analgesic effect, as has been hypothesized for the P3 animals.

The increase in mechanical sensory thresholds seen with the DA animals following codeine may again suggest that codeine or other metabolites of codeine are having the dominant analgesic effect. This is an unlikely explanation due to the fact that the effects between the two strains were so similar. SD rats are known to be able to produce morphine from codeine and it is would be surprising if they received very little or no analgesic benefit from the morphine produced, especially in light of the analgesic efficacy seen in animals of both strains following administration of morphine.

2.3.3 Carrageenan induced hypersensitivity

In inflammatory pain states widespread changes occur both peripherally and centrally leading to an altered perception of pain i.e. hypersensitivity. Carrageenan has been used in previous animal studies as a model for inflammation to simulate tissue injury, as may be seen following trauma or surgery.(Hargreaves K et al 1988; Kayser V et al 1987) In these current experiments, carrageenan produced inflammation at each of the age groups studied (P3, P10 and P21), characterized by redness and oedema of the hindpaw and a decrease in the mechanical threshold. This effect was maximal at two to three hours and stayed constant for the six hours which is in agreement with previous work.(Hargreaves K et al 1988; Kayser V et al 1987)

The decrease in mechanical threshold at 3 hours following carrageenan varied across the age groups, being smaller at P3 than at P10 and P21. There was no difference between the P10 and P21 animals. This increase in effect with age is in agreement with the previous studies and is probably explained by differences in the inflammatory response during development. The fact that the neonatal animals showed an inflammatory response and hypersensitivity suggest that in this state they provide a model for noxious stimulation using a mechanical stimulus such as von Frey hairs.

Mechanical hypersensitivity is very prominent in inflammation. Following inflammation, a state of hyperexcitability exists in sensory neurons in which activation of central cells by repetitive Aδ and C fibre input leads to amplification of non-noxious information from the periphery.(Woolf CJ et al 1999) This allows inputs that were previously ineffective to activate the neurons and previously effective inputs to be even more so. Another reason is that hypersensitivity is mediated by low threshold Aβ fibres which under most circumstances mediate innocuous stimuli.(Woolf CJ 1995) Aβ fibres can also increase the excitability of dorsal horn neurons during inflammation by the expression of substance P and BDNF.(Neumann S et al 1996)

NMDA receptors are also activated during inflammation and play a role in establishing central excitation.(Dickenson AH 1995) These processes develop considerably postnatally, with NMDA induced elevations in intracellular calcium markedly raised in the neonatal rat compared with the adult.(Hori Y et al 1994) Thus NMDA mediated effects such as wind-up and central sensitization maybe more prominent in the neonate and recent work has shown rat pups to be more sensitive to NMDA antagonists at earlier ages and that subunit composition of the NMDA receptor, which changes during development, may affect function.(de-Lima J 2001; Urch CE et al 2001)

In rats, there is evidence that the inflammatory process is immature at birth but undergoes considerable development during the first two post natal weeks. The ability of dorsal horn cells to display enlargement of receptive fields, increased spontaneous activity and enhanced responses to stimuli differs between the neonate and the adult.(Tornsey C et al 2000; Fitzgerald M 1995) "Wind-up" involving C fibre activity can only be demonstrated in a small proportion of dorsal horn cells at P10, but the activity then increases with age.(Fitzgerald M et al 2001) The substance P/NK1 system

is also immature in the first two postnatal weeks, which also affects central sensitization, but the mechanism is not known.(Fitzgerald M et al 2001) Using a chemical stimulus it has been shown that C fibres cannot produce neurogenic oedema until P11.(Fitzgerald M et al 1984) Part of the explanation may be due to low levels of substance P and other neuropeptides, but inflammatory mediators injected peripherally are unable to produce oedema until P3, suggesting receptor immaturity also plays a part.(Gonzalez DL et al 1993)

Testing in the contralateral paw showed no difference between the ages for the change in mechanical threshold but there was a small change from basleine at P3 and P10.The effects of inflammation are thought to be localised within the spinal cord but the results of the current experiments do not show this. Similar contralateral changes in threshold have been shown following peripheral nerve lesions and suggest that there are central signaling mechanisms that link both sides of the body, possibly via the system of commissural interneurons that is present in the spinal cord and brain stem, which may also be an explanation for the contralateral changes in thresholds seen in the current experiments.(Koltzenburg M et al 1999)

2.3.4 Effect of morphine and codeine on carrageenan induced hypersensitivity

Morphine reduced the hypersensitivity increasing the mechanical threshold above the pre-carrageenan baseline at all ages. However, the maximal effect was smaller at P3 than P10 and P21.This difference in effect with age could be due to the effect of development on the efficacy of opioids or may be a product of the differences in inflammation produced.

In all age groups the maximal effect with morphine is greater in the animals with carrageenan induced inflammation prior to the injection of the opioid. This phenomena of increased efficacy in pain states is well recognized in behavioural and electrophysiological studies and is thought to be due inflammation affecting the ability of CCK to regulate the action of opioids in the spinal cord and, possibly, increased descending inhibitory control involving alpha-2-adrenoreceptors during inflammation potentiating the effects of opioids.(Stanfa L et al 1995) The decreased effect of

morphine in the younger animals could potentially be explained by the reduced inflammatory response seen at these ages having less effect on CCK and alpha-2-adrenoreceptors modulation and thereby less potentiation of the opioid effects.

In comparison, codeine is ineffective at reversing the hypersensitivity at P3. In the P3 animals there is no difference between the effect seen with codeine and that seen with the control animals. At P10 and P21, as with all animals treated with morphine, the hypersensitivity was reversed and the maximal effect was greater than in animals from the same age groups treated with codeine that had undergone no prior intervention.

Once again morphine has an effect at all age groups whereas codeine does not. This again suggests that the decreased O-demethylase activity at P3 leads to decreased conversion of codeine to morphine compared with the older age groups and thus less anti-nociceptive effect seen with codeine.

The results from testing the contralateral paw of each of these animals showed a similar pattern. The maximal increase in vFh threshold demonstrated was smaller than that seen in the ipsilateral paw for all animals following both codeine and morphine but larger than that seen in the animals who had not had inflammation induced, apart from in the P3 animals treated with morphine. This again shows the increased efficacy of opioids in the presence of ongoing inflammation, with the smaller differences seen at P3 possibly explained by the immature inflammatory response seen at that age. However, these results differ from previous work that showed the effects of opioids in the contralateral paw are not altered in the presence of inflammation and suggested that the changes leading to enhancement of opioid effects in the presence of inflammation are localised.(Stanfa L et al 1995) These current results may suggest that whilst the majority of the changes during inflammation are probably localized, other changes may also be occurring at supraspinal levels that could affect both sides of the body.

2.3.5 Effect of naloxone

Naloxone is a potent opioid receptor antagonist and was used to investigate whether the effects of codeine and morphine were opioid mediated The experiments looking at the

effect of codeine and morphine on carrageenan were repeated at P3 and P10. However, on this occasion, naloxone and the opioid were given simultaneously at 3 hours.

In the P3 animals treated with codeine no change in effect was seen. However, in the P10 animals for both opioids and the P3 animals treated with morphine there was no increase in threshold for around 45-60 minutes and the overall effect seen was smaller. Naloxone has a shorter half-life and duration of action than both codeine and morphine.(Dolhery C 1999) The delay in response and overall decreased response is probably due to antagonism of opioid action. Thus it can be concluded that the effects seen with both codeine and morphine are due to activity at opioid receptors and that the lack of effect in P3 animals treated with codeine in all the experiments is due to a lack of opioid action. Previous studies showing the conversion of codeine to morphine is wholly or partly responsible for codeine analgesia suggest that the lack of opioid effect seen in the P3 animals given codeine in the current experiments is probably due to a lack of morphine produced.

2.3.6 Differences in opioid effects across the age groups

Apart from in the P3 animals following codeine, there was a significant response seen with both opioids, though some variations in effect were seen between the age groups. Previous studies have also shown differences in the effect of morphine during development though no consistent pattern has been identified.

There are many potential explanations for the differences in opioid effects seen during development:

Opioid receptor distribution. The distribution, and relative proportions, of the different opioid receptors in the brain and spinal cord changes during development. Mu receptors increase in density up to P7 and then gradually decrease to P21. Kappa and mu receptors are in the majority at P6, but delta receptors are low in number at birth and rise to a maximum by P21. In the adult mu and delta receptors predominate.(Rahman W et al 1998; Attali B et al 1990; Leslie FM et al 1982) However, the functional sensitivity of mu receptors to morphine was greater in P21 rats than P14 rats despite receptor

density being greater at P14. Thus receptor binding density does not necessarily predict function and maybe other factors are important for function such as site and receptor coupling.(Rahman W et al 1998)

The location of opioid receptors changes during development and it is possible that differences between adults and neonates may also affect the analgesic action of opioids In the adult mu receptor sites are concentrated in the superficial layers of the dorsal horn but at P1 both deep and superficial layers have high densities of receptors, which reach a peak at P4 before decreasing to adult levels and distribution.(Rahman W et al 1998) The decreased opioid effects seen in younger animals despite high receptor density may be due to the receptors identified in the neonatal period not being functional until they have reached their adult destinations.(Rahman W et al 1998) In adult rats mu receptors are situated both pre- (primarily on C fibres) and post-synaptically (on nociceptive specific interneurons).(Lombard M-C et al 1989) Little is known about the pre and post synaptic distribution of opioid receptors in younger animals. How these developmental changes in opioid receptor affect analgesic efficacy is unknown and further work is required before any conclusions can be drawn.

Opioid receptor development. Mu, delta and kappa opioid receptors are members of the superfamily of G-protein (guanine nucleotide-binding protein) coupled receptors. Each contains seven sequences of peptides, which take the form of membrane-spanning α helices. Above and below the membrane are peptide loops, the second and third cytoplasmic loops, providing the G-protein coupling site.(Pleuvry BJ 1997) Cloning of mu, delta and kappa has shown they share a high degree of sequence homology, with approximately half of the residues being identical.(Reisine T et al 1993)

Mu and delta receptors (and probably kappa) inhibit adenyl cyclase by activation of Gi (inhibitory) proteins, which reduces the production of cyclic adenosine monophosphate (cAMP). This leads to inhibition of calcium entry through voltage-sensitive channels and stimulates potassium efflux from the cell, resulting in hyperpolarisation and modulation of neurotransmission.(Greiff J et al 1994)

Altered opioid effects may also arise from differences in receptor dynamics and second messenger systems. G-protein coupling and activity are known to change during

postnatal development in rats which could then affect subsequent cellular effects.(Windh RT et al 1995)

The blood brain barrier. The BBB acts to control the solute content of the cerebral extracellular fluid (ECF) by restricting the movement of solutes from the systemic circulation to the cerebral ECF. The barrier is maintained by the presence of tight intercellular junctions between capillary endothelial cells. The presence of opioid in the CSF and their proximity to opioid receptors in the CNS is of critical importance to the pharmacological effects of opioids. Thus developmental differences in the strusture and function of the BBB could have an effect of the amount of opioid present in the CNS and thus on efficacy.

This, however, has not been shown. In the neonatal rat, physiological and histological study has shown that the BBB is intact at birth.(Butt AM et al 1990) Also in further work involving M-6-G, the function of the BBB did not alter with dose or age in guinea pigs.(Murphey LJ et al 1994) Autoradiographic studies also show that the amount of morphine binding in the spinal cord following systemic administration is equivalent in SD rats at P3, P10 and P21.(Marsh DF 1997) However, a recent study has shown that the removal of morphine from the CSF is via an active transport system located in the BBB and down regulation of this transport system leads to increased analgesic effect.(King M et al 2001) Nothing is known about the development of the activity or function of this transport system.

Neurological development. As has already been reviewed earlier in this thesis, during development the peripheral and central nervous system undergo tremendous changes, with respect to pain processing and reflex activity, at all levels. Although much is known about these changes, the picture is far from complete. These differences between stages of development may have a significant effect not only on the response seen to different stimuli but also on how that response is modulated by different chemicals or interventions e.g. opioids.

Pharmacokinetics. How the body handles a drug changes with age and is an important cause of variation in the efficacy of drugs. In humans it is known that the pharmacokinetics of morphine changes during the neonatal period. The clearance and

half-life both decrease during the neonatal period and can reach adult values anywhere between 2 weeks and 2 years. Though the volume of distribution is relatively constant with age.(Kart T et al 1997; McRorie TI et al 1992) The metabolism of morphine and relative production of its glucuronide metabolites also changes during the neonatal period.(Hartley R et al 1994; Hartley R et al 1993; Chay PCW et al 1992; Bhat R et al 1992; Choonara IA et al 1989) These differences will potentially cause changes in efficacy, though more work is required in this area. The neonatal pharmacokinetics of codeine has not been studied in humans.

The pharmacokinetics of both codeine and morphine have not been studied during development in rats. Though it seems logical to assume that differences during development could potentially have an effect on efficacy.

Development of enzyme activity. The activity of the cytochromes P450 and the expression of different cytochromes P450 changes with development.(Oesterheld JR 1998) The changes in activity of rat O-demethyase activity, and its possible consequences on the efficacy of codeine, are a potential explanation for the decreased efficacy seen in the P3 animals and could also play a part in variations in efficacy between other age groups. Changes in enzyme activity and affinity for the substrate is also dependent on the phospholipid composition of the membrane which changes between fetal and adult microsomes in both humans and rats.(Kapitulnik J et al 1986) Differences in the activity of the enzymes involved in the metabolism of codeine and morphine may also play a role in altering their efficacy during development.

Other possible explanations for the differences in opioid effects seen during development may include problems with experimental design such as the difficulty in achieving equivalent noxious stimuli at different ages and developmental differences in the inflammatory response due to differences in neurological development between the ages. Also practical difficulties in behavioural testing with young animals and the different behavioural tests employed may produce conflicting results.

2.3.7 Summary

Overall the results from these experiments show there is developmental regulation of the efficacy of codeine in rats and that an opioid effect is responsible for codeine efficacy. Codeine lacks efficacy in the P3 animals which, though there are many possible reasons for differences in opioid effect during development, a lack of production of morphine from codeine at this age due to low activity of the enzyme converting codeine to morphine may be important.

Although the extrapolation of animal work into humans must be done with caution these results have a potential significance in humans. It is known that the activity of CYP2D6 is low in human neonates and has only reached 25% of adult levels by five years of age.(Jacqz-Aigrain E et al 1992) Low activity of the enzymes converting codeine to morphine seems to lead to decreased efficacy in rats and if the same is true in humans we are potentially treating a group of patients with an ineffective drug. Pain scoring in infants and neonates is notoriously difficult and any under treatment of pain may go undetected.

More work in this area is required. Activity of the enzyme could be looked at indirectly by taking blood from the SD animals at each of the ages to measure plasma morphine and metabolite levels following codeine and morphine administration (see Chapter 3).

There is also developmental regulation of the efficacy of codeine in DA rats with responses to codeine seen at P10 and P21 but not at P3 and P63-70. This has important implications for its use as a model for the PM phenotype. Measurement of plasma morphine levels in DA animals could also be undertaken to see if morphine is produced at these ages and thus provide a possible explanation for the effects seen. Studies with naloxone could also be designed using DA rats to investigate whether there is an opioid mechanism for the analgesic effects seen with codeine.

To confirm that it is the conversion of codeine to morphine that is important for efficacy during development these experiments could be repeated using pharmacological blockade of the O-demethylase reaction with competitive blockers of CYP2D1 such as quinine.

Also well-designed clinical trials are also required in human neonates and infants to assess the efficacy of codeine at these age groups, during which blood could also be taken to quantify morphine production. Though again the accurate quantification of pain in these age groups makes the design of such experiments difficult.

CHAPTER 3. PLASMA MORPHINE SAMPLING

3.1 Introduction

The behavioural experiments presented in this thesis suggest that there is developmental regulation of the efficacy of codeine in both SD and DA rats. In an attempt to try and answer some of the questions posed by the results from the behavioural experiments measurement of plasma morphine and morphine metabolite levels was undertaken from animals of both strains following codeine and morphine administration, using the same age groups. The measurement of morphine and its glucuronide metabolites has been refined in recent years with the development of more specific and sensitive assays using High Performance Liquid Chromatography (HPLC).

Codeine analgesia is thought to be due to the O-demethylation of codeine to morphine. In the SD strain the neonatal pups (P3) show decreased effects of codeine compared with older animals and animals of all ages treated with morphine. One possible explanation may be that low enzyme activity is responsible for the decreased efficacy of codeine. O-demethylase activity in female SD rats at P3 is approximately one-fifth of that at P10, P21 and adult animals.(Matsubara T et al 1986) Measurement of plasma morphine, and morphine metabolite levels, provides an indirect measure of enzyme activity, in as much as it will show whether morphine is produced, and relative amounts, after administration of codeine. However, plasma levels must be viewed with caution as direct correlation between plasma morphine levels and analgesic effect has not been shown during clinical trials in children given either intermittent dosing or intravenous infusions of morphine.(Kart T et al 1997a; Millar AJW et al 1987; Lynn AM et al 1984; Nahata MC et al 1984)

DA animals are used as a model of PMs of codeine in animal experiments because they lack the CYP2D1 enzyme that is said to be analogous to the CYP2D6 enzyme in humans. In previous work adult DA animals showed little response to codeine and in these experiments both P3 and adult animals also show reduced effects of codeine. However efficacy was shown at P10 and P21 following codeine. There are potential explanations for this difference and measurement of plasma morphine levels will show

whether morphine is produced following codeine administration by DA rats, at any or all of the ages studied.

3.1.1 Morphine and its metabolites

Morphine is mainly metabolised by glucuronidation to morphine-3-glucuronide (M-3-G) and morphine-6-glucuronide (M-6-G), though a number of other lesser metabolites have been identified e.g. sulphates, normorphine, normorphine-3-glucuronide, morphine-3-6-glucuronide and codeine. Metabolism occurs mainly in the liver but also occurs in the kidney, CNS and intestines. Excretion is mainly via the urine with over 90% of a dose of morphine being excreted via this route with some also found in bile and faeces.(McQuay H et al 1997)

Glucuronidation is facilitated by the action of uridine diphosphate glucuronosyl-transferases (UDPGTs), a family of isoenzymes, of which 11 different forms have been identified, and full function of these enzymes appears to develop at different rates postnatally(Hartley R et al 1995) It has been suggested that two isosymes of UDPGT are responsible for the metabolism of morphine though recent work has identified a single UDPGT (UGT2B7) that is capable of both –3 and –6 glucuronidation.(Coffman BL et al 1997; Coughtrie MWH et al 1989) A recent systematic review suggests that the formation of M-3-G and M-6-G is highly correlated which supports the idea of a single UDPGT for the glucoronidation of morphine.(Faura CC et al 1998) However, the ratio of M-3-G:M-6-G in neonates is variable and this may reflect the action of more than one isosyme.(Kart T et al 1997b)

M-3-G

M-3-G is the predominant metabolite, has no analgesic activity and does not bind to opioid receptors though it does have CNS stimulatory effects not mediated through opioid receptors.(Wolff T et al 1995; Christensen CB et al 1987; Schulz R et al 1972) It has been thought that M-3-G is antagonistic to the analgesic and respiratory depressant effects of morphine and M-6-G.(Gong QL et al 1992; Smith MT et al 1990) However, there is good evidence that there is no direct pharmacological antagonism of either

morphine or M-6-G by M-3-G, and that the antagonism reported is best thought of as functional.(Penson RT et al 1997; Faura CC et al 1996; Hewett K et al 1993) Anatagonism of the effects of morphine by M-3-G is unlikely given the knowledge that M-3-G does not bind to opioid receptors and the fact that morphine works in clinical practice despite the presence of M-3-G. For example, in patients with renal failure M-3-G accumulates due to the decreased excretion. Thus it would be expected that morphine would be less effective in these patients. However, the opposite is true and morphine doses have to be decreased or dosage intervals increased to avoid over dosing.

M-6-G

M-6-G has been shown to have analgesic properties. In mice it was shown to be 3-4 times more potent as an analgesic than morphine following subcutaneous injection and 45 times more potent following intracerebroventricular injection. In rat it has also been shown to be 10-20 times more potent than morphine following intrathecal administration.(Sullivan AF et al 1989; Pasternak GW et al 1987; Shimomura K et al 1971) Further studies in both humans and animals have shown it to be a potent mu agonist. In one study comparing intrathecal M-6-G and morphine in cancer patients showed that following administration of the test drug, opioid requirements in the following 24 hours were less after M-6-G than morphine. (Gong QL et al 1991; Hanna MH et al 1990; Hand CW et al 1987) Morphine is probably partly dependent on M-6-G production for its activity. In experimental studies in human volunteers the respiratory depressant effects of M-6-G have been shown to be less than those of morphine. One study showed both morphine and M-6-G depressed respiration but the effect was significantly less following M-6-G, whilst another study showed no depressant effect with M-6-G at increasing doses.(Thompson PI et al 1995; Peat SJ et al 1991)

3.1.2 Morphine and metabolite levels

Plasma

Studies in humans following oral dosing have shown that the levels of M-6-G are similar though higher than those of morphine and about one-tenth those of M-3-

G.(Jones SF et al 1988; Svensson J-O et al 1982) A comparison of intravenous and oral doses of morphine found that the proportions metabolised to M-6-G and M-3-G were the same irrespective of the route of administration and that the terminal half lives of both morphine and its metabolites were long.(Hasselstrom J et al 1993)

Due to the presence of these metabolites with differing pharmacological properties a common method used to help with the understanding of the pharmacology of morphine has been the comparisons of metabolite:morphine and metabolite:metabolite ratios.(McQuay H et al 1997) Data from a large number of trials looking at plasma morphine and metabolite levels have been used to give an overall picture of these ratios in humans.(Faura CC et al 1998; McQuay H et al 1997)

Following single oral doses of morphine the median M-6-G:morphine ratio is 5.4 (range 0.96-11) and the median M-3-G:morphine ratio is 25 (9.9-56). These levels are similar to those found after multiple oral dosing. However, the levels following a single intravenous dose are about six times lower at 0.6 (0.29:2) for M-6-G:morphine and 6.1 (2.8-11.1) for M-3-G:morphine, reflecting the first pass metabolism which affects the oral dose only. As already discussed the formation of M-3-G and M-6-G is highly correlated and the ratio of M-3-G:M-6-G remained relatively constant over a large number of studies with a mean of 6.3 (0.5-13.3). However, as well as route of administration, all these ratios could be affected by other factors such as age and organ impairment.(Faura CC et al 1998; McQuay H et al 1997)

CSF

Two studies using rats have looked at morphine and its metabolites in the CNS. The first showed maximum CSF levels of morphine and its metabolites were reached after 45 minutes following subcutaneous injection and concluded that the glucuronide metabolites were able to cross the blood brain barrier at the same rate as morphine but in larger quantities. The other study looked at M-6-G and morphine following subcutaneous injection and found that M-6-G produced better analgesia assessed by

behavioural testing and concluded that this was due to higher levels of M-6-G in plasma and the brain.(Stain F et al 1995; Barjavel M et al 1994)

In humans one of the reviews discussed above also analysed the studies of morphine and metabolite levels to compare plasma and CSF levels.(McQuay H et al 1997) Although there have been fewer studies looking at CSF levels it has been shown that M-6-G does cross the blood brain barrier into the CSF. M-6-G may also cross in amounts 2-4 times greater than M-3-G. The median CSF:plasma ratio for M-6-G it is 0.044 (0.008-0.27) and for M-3-G it is 0.014 (0.004-0.1). These figures are higher with multiple dosing and following epidural or intrathecal administration.

How much M-6-G contributes to the analgesia of morphine is still a matter of debate. The fact that it is present in the CSF and is known to have analgesic properties suggests that it will have a significant effect. Its concentration in the CSF is 0.12 times that of morphine, but if the potency of M-6-G is 10-20 times greater than that of morphine in humans, as seen in rats (Sullivan AF et al 1989; Pasternak GW et al 1987), it could make a significant contribution to analgesia. However, there have been no clinical trials to demonstrate this.

3.1.3 Effect of development on morphine metabolism

In humans it has been suggested that the glucuronidation pathway is present but immature at birth and continues to develop after the neonatal period.(McRorie TI et al 1992; Choonara IA et al 1989) It is well established that term neonates, infants and young children are capable of metabolising morphine to M-3-G and M-6-G.(Choonara I et al 1992; Cederholm I et al 1990; Choonara IA et al 1989; Shelly MP et al 1986) Studies in preterm infants have shown diverging results regarding the presence of metabolites.(Hartley R et al 1994; Hartley R et al 1993; Chay PCW et al 1992; Bhat R et al 1992; Choonara IA et al 1989) Sensitivity of the assay and route of administration may have affected the results in those studies where metabolites were not detected in some babies. However, a weak, but significant, correlation was found between glucuronidation capability and gestational age in one study and another study, where

babies did not produce metabolites, involved babies that were younger and lighter.(Hartley R et al 1994; Bhat R et al 1992)

The ratio of M-3-G to M-6-G decreases with decreasing birthweight and in comparison with adults using meta-analysis, studies involving neonates show the ratio is lower in neonates (< 1 month), 3.1 (2.6-3.5), compared with that in children (>1 month), 6.2 (2.3-20), and adults, 6.3 (0.5-13.3).(Faura CC et al 1998; McRorie TI et al 1992) Thus there is relatively more M-6-G present at these younger ages and thus a potentially greater analgesic effect. The ratios of M-3-G:morphine and M-6-G:morphine are lower in neonates than children confirming that neonates have a lower metabolic capacity than older children.(McRorie TI et al 1992; Choonara IA et al 1989) Comparing these results with further work in term neonate and infants shows M-3-G:morphine ratios increase with age from the premature neonate through to children.(Choonara I et al 1992) This lower metabolic capacity has been used to explain the longer duration of action of a single dose of opioid in a neonate(Kart T et al 1997b)

The pharmacokinetics of morphine also varies with age. Though volume of distribution would appear to be similar across all age groups, half-life increases with decreasing age and clearance is decreased in premature and full term neonates with adult values being achieved at around two months, though there was a large amount of variability between results and adults levels could be reached as soon as 2 weeks or as late as 2 years. This could potentially lead to the accumulation of morphine and its metabolites at these younger ages.(Kart T et al 1997b; McRorie TI et al 1992)

3.1.4 Morphine and metabolite levels in rats

Morphine

Work in rats has shown that M-3-G is the dominant metabolite. Adult male SD rats given intra-peritoneal morphine 4mg/kg showed peak plasma morphine levels (1060 ng/ml +/-133) around 8 minutes after the injection before declining rapidly to be about one fifth of these levels by 80 minutes. Whereas for M-3-G peak plasma levels (2902 +/-629) occurred at 30 minutes and stayed elevated up to 40 minutes before declining.

Although exact levels are not given, the M-3-G:M ratio can be estimated at around 3-4:1.(Zheng M et al 1998) M-6-G was not detected in any of the rats at any time. This is in accordance with results from in vitro studies in rats.(Coughtrie MWH et al 1989; Rane A et al 1985)

Another study involved the intraperitoneal injection of morphine 1mg/kg to adult male SD rats. Peak plasma morphine levels were reached at around 9 minutes (200 ng/ml +/- 30), which then declined to approximately one quarter of these levels by 60 minutes. Again M-3-G was shown to be the major metabolite with peak plasma levels reached at 20 minutes. The ratio for AUC M-3-G:M was calculated to be 2.0 +/- 0.5. In this study M-6-G was detected in some rats but only in negligible amounts.(Hasselstrom J et al 1996) No limit of detection for M-6-G was given in this study compared with a limit of 26 ng/ml in the previous study.(Zheng M et al 1998)

Species and sex differences in the metabolism may also affect the glucoronidation of morphine.(Hasselstrom J et al 1996; Belknap JK et al 1991; Blanck A et al 1990) The above data were only obtained from male animals and I am unaware of any similar studies involving DA rats. To my knowledge there is also no work looking at plasma morphine and metabolite levels following administration to rats other than in adults.

Codeine

Studies in male rats have shown that about half of a dose of codeine is metabolised to morphine and that morphine is present in the CNS following codeine administration.(Dahlstrom B et al 1976; Yeh SY et al 1969) A study investigating morphine levels in adult male SD rats following intravenous codeine doses of 1, 1.5, 3 and 4mg/kg showed that at all doses codeine is rapidly metabolised to morphine with a time to maximum morphine concentration of between 2 and 6 minutes, with plasma levels declining over time, by one hour the levels were approximately one-fifth of the maximum levels. Maximum levels of morphine increased with increasing dose (5.8 – 76.8ng/ml) and the amount of codeine metabolised to morphine also increased with dose. The glucuronide metabolites of morphine were not measured.(Shah J et al 1991)

Another study by the same authors using adult male SD rats looked at plasma morphine levels following intravenous codeine 3mg/kg and oral codeine 5mg/kg. By both routes of administration metabolism to morphine was rapid with the time to maximum morphine concentration being 4.2 minutes intravenously (24.8ng/ml) and 6.5 minutes orally (68.7ng/ml). Again plasma levels declined over time and by 60 minutes were approximately one quarter of maximum levels in both groups.(Shah J et al 1990)

Further work comparing codeine metabolism in liver microsomes from adult female SD and DA rats showed that the maximum velocity of the O-demethylation reaction was twice as high in the SD rats and the intrinsic clearance of codeine to morphine was 10 times lower in the DA rats. This study also demonstrated inhibition of codeine O-demethylation in SD rat liver microsomes by the use of competitive inhibitors of enzyme activity such as quinine.(Mikus G et al 1991)

Once again I am unaware of any work relating to plasma morphine and metabolite levels in rats after codeine administration other than in adults. There is also the potential for sex and species differences to affect the metabolism of codeine to morphine in rats and it is known that O-demethylase activity in SD rats is decreased in adult female rats compared with adult males.(Matsubara T et al 1986)

Aims

1. To measure plasma morphine and morphine metabolite levels after administration of intra-peritoneal codeine, morphine or saline in SD and DA rats at each of the age groups studied in the behavioural experiments.
2. To measure these levels at the time of peak effect of the opioid taken from the data recorded during the behavioural experiments.
3. To study the changes in plasma levels with time in adult animals, levels will also be recorded at other time points
4. To see if there is a correlation between plasma levels and effect. The change in vFh threshold from baseline will be recorded immediately prior to collection of blood for measurement of levels

Hypotheses

1. Morphine and M-3-G will be produced by SD animals at all age groups following both codeine and morphine administration but metabolism will be less in the P3 animals treated with codeine.
2. Morphine and M-3-G will be produced by DA animals at all age groups following both codeine and morphine administration but metabolism will be less in the P3 and adult animals treated with codeine.
3. The metabolism of both codeine and morphine will be affected by the age of the animal.
4. There will be no correlation between plasma morphine levels and effect at any age group.

3.2 Materials and methods

All rats were kept fed, watered and with their mother until the start of each experiment. Female Sprague SD rats were obtained from the animal house at University College, London. Experiments involved comparisons of animals aged P3 (mean weight 9.05g ± 1.11), P10 (mean weight 22.28g ±2.85) and P21 (mean weight 51.93g ±5.72).

3.2.1 Injection techniques and opioids studied

In all the experiments the drugs were again given via the intra-peritoneal route using the same techniques, equipment, volumes and concentrations as described in Chapter 2. The doses of opioids used for comparison in all the experiments were once more codeine 10mg/kg and morphine 2mg/kg diluted to a volume of 10ml/kg.

3.2.2 Studies of mechanical sensory thresholds

These experiments involved applying a low intensity mechanical stimulus to the dorsal surface of the left hind paw using von Frey hairs as described in Chapter 2. At the beginning of each experiment a baseline vFh threshold was taken on each animal at time t_0. The opioid to be tested was then given by intra-peritoneal injection. From the data obtained during the experiments in Chapter 2, an estimate of the time to peak effect was made. For animals at all three age groups receiving morphine this was 45 minutes. For animals receiving codeine the time to peak effect was 30 minutes for P3 pups and 45 minutes for P10 and P21 pups. At the appropriate time for each animal and drug a repeat vFh threshold was taken. Blood was then withdrawn for the measurement of plasma morphine and its glucuronide metabolite levels. During these experiments animals were chosen at random to receive intra-peritoneal injections of saline to act as controls.

3.2.3 Blood sampling

The circulating blood volume of a rat is approximately 55 – 70 ml/kg.(Wolfensohn S et al 1998) Thus, for example, in a P3 animal weighing 10g the blood volume is around 0.55 – 0.7 ml. The measurement technique used for estimation of plasma morphine and its glucuronide metabolite levels (see section 3.2.4) required a plasma volume of at least 0.4ml. Hence, especially in the smaller animals, blood could only be taken at one time point, as this sampling was a terminal event. Therefore the estimated time to peak effect, as described above, of each drug was chosen as the time for blood sampling.

The attempted removal of essentially as much of the circulating volume as possible from P3 and P10 animals proved to be very difficult. A number of different anaesthetic and blood sampling techniques were tested. These included anaesthesia using carbon dioxide, halothane in 100% oxygen and intra-peritoneal injection of pentobarbitone. Attempts at blood withdrawal were made by indirect cardiac stab, thoracotomy plus direct cardiac stab, directly cutting open the left ventricle, cannulation or needle puncture of major venous or arterial vessels and attempted perfusion using air to push blood out of the circulation.

Although no single technique was found to be consistently reliable, initial pilot studies showed most success was achieved by anaesthetising the animal in a mixture of halothane 2 – 4% in 100% oxygen and withdrawing the blood via direct cardiac stab following exposure by thoracotomy. This technique was then used during all subsequent experimentation in all three age groups. Blood was withdrawn using a 0.5ml (insulin) syringe and a 30G needle for the P3 pups, a 1ml syringe and 25G needle for the P10 pups and a 2ml syringe with a 23G needle for the P21 animals. The 0.5ml and 1ml syringes were coated with heparinised saline (heparin 5units/ml, University College Hospital Pharmacy) to prevent the blood clotting.

Once the blood had been withdrawn it was transferred into either a 1ml or 2 ml lithium heparin blood collection tube, which was then placed in a centrifuge and spun at 3,000 revolutions per minute for 10 minutes. The plasma was then carefully pipetted, so as to obtain as much plasma as possible, into plastic storage tubes. These were then stored in

a –70°C refrigerator until plasma morphine and its glucuronide metabolite levels were measured.

Sampling from SD adult animals

Female SD P63-70 (i.e. adult, mean weight 232.4g ±24.48) rats were obtained from the animal house at University College, London. All animals were kept fed and watered until the start of each experiment.

Each animal was initially injected with the opioid to be studied at a volume of 10ml/kg. Blood was then withdrawn from each animal on two different occasions selected at random from four different time points – 10, 20, 30 and 45 minutes. Each time 1.5 – 2ml of blood was withdrawn. It was felt that withdrawing more than two samples from an individual animal would reduce the circulating volume to such an extent that there could have been an effect on the pharmacokinetics of the drug. Blood was withdrawn using an indirect cardiac stab with the animal anaesthetised using 2 – 4% halothane in 100% oxygen using a 2ml syringe and a 23G needle. The plasma was then separated off and stored as described.

Mechanical sensory threshold testing was not performed on these animals, as the use of repeated episodes of anaesthesia may have affected the measurement of the thresholds.

Studies with the DA strain

Female DA rats were purchased from Harlan OLAC, UK and were transported to the animal house at University College, London prior to each experiment with sufficient time to allow for acclimatization. Experiments involved comparisons of animals aged P10 (mean weight 14.97g ±1.32), P21 (mean weight 41.55g ±2.99) and P63-70 (mean weight 152.67g ±9.63). All animals were kept fed and watered, and with their mother in the case of the rat pups, prior to the start of each experiment.

Experiments involving P10 and P21 pups again involved von Frey hairs as the mechanical stimulus and were the same as those described for SD P10 and P21 pups. It was decided not to attempt morphine sampling from DA P3 pups as results from SD P3 pups had shown that it was not possible to obtain enough blood to give meaningful results and the DA P3 pups were slightly smaller than the SD P3 pups (8.86g vs 9.47g). Blood was once more withdrawn at the time of peak response, which was estimated to be 45 minutes for both codeine and morphine from the data obtained during the experiments described in Chapter 2. The methods of blood collection and plasma separation and storage were again the same as those described for SD P10 and P21 pups.

Morphine sampling from adult animals was undertaken using the methods described for SD adults. However, this time due to the smaller number of animals available, only two time points were chosen – 20 and 45 minutes. Once again mechanical sensory threshold testing was not performed on these animals, as the use of repeated episodes of anaesthesia may have affected the measurement of the thresholds.

Termination

At the conclusion of each experiment all the animals were terminated by the intra-cardiac, if the chest had been opened by thoracotomy, or intra-peritoneal injection of Pentobarbitone (University College Hospital Pharmacy) in a dose suitable to produce euthanasia at each age group.

3.2.4 Measurement of morphine and its glucuronide metabolites

Measurement of morphine and its glucuronide metabolites from all the plasma samples obtained was performed by High Performance Liquid Chromatography (HPLC). Analysis was carried out by The Children Nationwide Research Centre, which is based at the Institute of Child Health, London, using a method and equipment designed and built under the direction of Dr Jonathan De-Lima and Ines Kastner. Analysis of all the samples was performed by Ines Kastner.

Morphine, M-3-G and M-6-G concentrations were determined simultaneously, by solid phase extraction followed by reverse-phase ion-paired chromatography with electrochemical and fluorescence detection as described by Joel, Osborne and Slevin.(Joel SP et al 1998) This technique was updated to include newer disc based extraction devices which are suited to small sample volumes and may improve the sensitivity of the whole analytical process (SPEC Ansys UK, 1994). The method was designed initially to measure plasma samples of 0.5ml but refinement of the technique has allowed plasma samples of 0.35ml to be analysed.

The overall process is comprised of three separate phases:

1. Extraction:

   Plasma samples are purified using a SPEC disc (C18AR, Ansys, UK). The extraction is then carried out using an ASPEC XLI automated sample and injector (Gilson, UK). This solid phase extraction comprises of four steps:

   - Condition – SPE disk columns containing 15mg of C18AR are conditioned with 0.5ml acetonitrile, followed by 0.25ml of water. This activates the C18 chains of the bonded phase to enable them to interact with the sample matrix.
   - Load – Human plasma samples (0.5ml) are diluted in a ratio of 1:2 with a buffer, 500mM ammonium sulphate (pH 9.3). 1ml is then loaded onto the SPE column.
   - Wash – Interfering matrix compounds are removed from the sorbent with small volumes of wash solutions. This is done using 0.25ml 5mM ammonium sulphate as the buffer followed by 0.1ml of water.
   - Elution – The analyte produced is then desorded with 0.55ml of 10% methanol, 50mM sodium dihydrogenorthophosphate1-hydrate that weakens analyte-sorbent interactions. This elutate is then compatible with the mobile phase of the HPLC.

   0.25ml of elutate is then filtered through a 0.45μm Millipore filter and injected onto the HPLC.

2. Chromatography:

   Separation of analytes using chromatography can be influenced by a number of facors:

   - Stationary Phase – i.e. the column. This method used a 25cm silica based column, Reverse Phase (RP)-select B (5μm) LiChrospher 60, purchased from BDH/Merck UK.
   - Mobile Phase – the composition of the mobile phase used was 20% acetonitrile + 50mM phosphate/0.75mM DDS (lauryl sulphate, ion-pair reagent) + water, overall pH of 2.1. The mobile phase was recycled.
   - Temperature – the system was run at a temperature of 35°C.
   - Flow rate – a flow rate of 1ml/min was used.

3. Detection:

   Morphine and M6G were measured using electrochemical detection using a Coulochem 5200A detector. M3G however, is not electrochemically active, and was measured using a Gilson 122 ultra violet fluorescence detector.

Time for extraction and analysis was 25 minutes per sample with method design allowing samples to be run in batches. The limits of detection varied between batches of samples analysed (see below). Mean extraction efficiency was >90% for each analyte with a variability between samples of 8%.

Recordings were presented as chromatograms, which were saved electronically. The peak heights were calculated using the software program "UNIPOINT" and the data the transferred into an EXCEL worksheet to calculate averages and standard deviations.

Problems encountered during HPLC analysis

Due to the difficulties with blood collection, especially in the smaller animals, already described and occasional analysis problems with the HPLC process not all the animals tested provided a result. The table of results for each set of animals studied includes only those animals that yielded a result of some kind.

An attempt was made to refine the analysis process of the HPLC to allow quantification of small volume plasma samples, down as low as 0.1 – 0.2ml. However, this proved unsuccessful and only plasma volumes of 0.4ml and above could be successfully quantified.

The samples were analysed in three batches and between these batches the limits of detection changed for M-3-G and M-6-G. For the M-6-G the limit was 10ng/ml in the first two batches and 20ng/ml in the third. For M-3-G the limit was 30ng/ml in the first batch and 60ng/ml in the subsequent two batches. However, in all three batches the limit of detection for morphine was 5ng/ml.

3.2.5 Analysis

All data was handled electronically and calculations performed using either Microsoft Excel 2000 or Graphpad Prism 3.0. Comparisons between continuous variables were made using either ANOVA with Bonferroni post test corrections or t-tests, depending which test was appropriate. Correlation between independent variables was tested with Pearsons correlation coefficient. For all comparisons $p<0.05$ was considered significant.

3.3 Results

Blood was taken from SD and DA rats at different ages (P3, P10, P21 and P63-70) for measurement of plasma morphine and morphine metabolite levels following intra-peritoneal injection of opioid or saline. In the P3, P10 and P21 of both rat strains baseline vFh thresholds were recorded prior to injection of the test drug and the change in threshold was then measured immediately prior to morphine sampling.

3.3.1 Morphine sampling effects

For both strains the samples were taken 45 minutes after injection at all ages in pups receiving morphine and in the P10 and P21 pups receiving codeine. In the P3 animals receiving codeine the samples were taken at 30 minutes after injection. These times correspond to the time to peak effect for each drug at each age calculated from the results in Chapter 2. Data was analysed using one and two way ANOVA with Bonferroni post-test corrections. A p value of <0.05 was considered significant.

SD pups

Figure 17 shows the mean changes in mechanical threshold of SD rat pups at ages P3, P10 and P21 following the intra-peritoneal injection of morphine 2mg/kg, codeine 10mg/kg and saline.

Due to the difficulties with blood collection in the smaller animals the numbers of pups used in each group were:

SD	Codeine animals (n)	Morphine animals (n)
P3	23	20
P10	22	22
P21	10	10

Comparison of the behavioural effects of all animals shows a significant interaction between age and drug and a significant effect of age and drug dose, all with $p<0.0001$.

Effect of morphine

Morphine gives a consistent rise in mechanical threshold at all ages but the magnitude of the effect varies across the age groups. There is no difference in the effect between the P3 and P21 pups and the P10 and P21 pups. However, there is a greater effect in P10 pups compared with the P3 pups ($p<0.001$). There is also no difference between the effects seen in these pups and the mean peak effects seen in the equivalent pups studied in Chapter 2 at any of the ages i.e. showing reproducible analgesia.

Effect of codeine

As with the results for the SD animals in Chapter 2, P3 animals demonstrate a much smaller response to codeine than P10 and P21 animals ($p<0.001$). There is also a significant but smaller difference in effect between the P10 and P21 pups ($p<0.01$), with the P10 pups showing the larger response. However, there is no significant difference between the effects seen in these pups treated with codeine and the mean peak effects seen in the equivalent pups studied in Chapter 2 at any of the ages.

Effect of morphine vs codeine

In contrast to codeine, morphine is equally effective at all 3 ages. There is no significant difference between the response to codeine and morphine in either of the P10 and P21 groups but there is a smaller effect seen in the P3 animals treated with codeine compared with those treated with morphine ($p<0.001$).

Effect of saline

In each litter studied one animal was chosen at random to receive saline rather than opioid. In all these animals there was no change in the mechanical threshold from baseline in any of the age groups.

DA pups

Figure 18 shows the mean changes in mechanical threshold of DA rat pups at ages P10 and P21 following the intra-peritoneal injection of morphine 2mg/kg and codeine 10mg/kg. Due to the difficulties in collecting blood from the SD P3 pups it was decided not to attempt to sample from the DA P3 pups. For each dose of opioid and each age group n = 5 animals.

Effect of opioids

At both these ages the effects of codeine and morphine were similar. In both the P10 and P21 pups there was a consistent rise in mechanical threshold following morphine and codeine and there was no significant difference between the responses in these animals in either age group, between either drug or between the equivalent animals in Chapter 2.

Comparison between the strains

Comparisons of the effects of morphine between the two strains were similar but comparisons involving codeine showed an increased effect with the SD P10 pups. Following morphine there was no difference between the change in mechanical threshold seen in these experiments between the SD and DA pups. Also, following codeine there was no difference in effect seen in the P21 animals. However, a greater effect was seen in the SD pups at P10 ($p<0.01$).

3.3.2 Morphine sampling

Plasma samples were obtained from P63-70, P21, P10 and P3 SD animals and from P63-70, P21 and P10 DA animals as described in the methods section 3.2.3 and were analysed for measurement of plasma levels of morphine and its glucuronide metabolites

using HPLC by Innes Kastner and Jonathan De-Lima at The Institute of Child Health, London.

The data from these experiments is shown in tables 2-10. Where possible an average plasma level has been calculated for morphine and the morphine metabolites at each age group and each strain. Where a range has been quoted the mid-point of the range has been used e.g. for an M-6-G result of 5-15ng/ml, 10ng/ml is used as the value for the calculation. Data were analysed using unpaired t-tests or one-way and two-way ANOVA with Bonferroni post-test corrections, depending on which comparisons were being made, with $p<0.05$ considered significant.

SD P3 animals

Table 2 displays the results of analysis of plasma for morphine and morphine metabolite levels in SD P3 pups. Morphine and its metabolites were detected in animals receiving both codeine and morphine though, due to the small volumes of the samples in this age group, quantification was not possible.

SD P10 animals

Table 3 shows the plasma morphine and morphine metabolite levels for SD P10 pups. Again morphine and its metabolites were seen in both groups but not in the saline animals, though once more quantification was not possible in all animals. Morphine levels were not different between the two groups but the levels of M-3-G were higher in the codeine animals ($p<0.01$) and M-6-G was detected in the codeine animals but not the morphine animals, though only at low levels.

SD P21 animals

Table 4 shows the plasma morphine and morphine metabolite levels for SD P21 pups. Morphine and its metabolites are seen in both groups. The plasma levels of morphine are not different between the two groups but the levels of M-3-G are significantly higher

in the codeine animals ($p<0.01$) and M-6-G is only detected in the codeine animals, though again only at low levels.

DA P10 animals

Table 5 shows the plasma morphine and morphine metabolite levels for DA P10 pups. Morphine and morphine metabolites are produced by both the morphine and codeine groups of animals but again as sample volumes were small and it was not possible to quantify most of the results, and it is not possible to make quantative comparisons between the two groups.

DA P21 animals

Table 6 shows the plasma morphine and morphine metabolite levels for DA P21 pups. Morphine and its metabolites are seen in both groups. The plasma levels of morphine are not different between the two groups but the levels of M-3-G are significantly higher in the codeine animals ($p<0.01$) and M-6-G is not detected in the either group of animals.

SD Adult animals

Table 7 shows the plasma morphine and morphine metabolite levels for SD adult animals following morphine 2mg/kg, and table 8 shows the comparative results for SD adult animals following codeine 10mg/kg. Figure 19 shows a graphical comparison between the two groups of animals for plasma morphine and M-3-G levels over time.

In the animals treated with morphine, two results from the morphine levels at 30 minutes and one result at 20 minutes have been treated as biological outliers as they are very different compared with other results at those time points and with results at surrounding time points.

Comparing morphine levels between the two groups shows significant interaction of time and drug ($p=0.0001$) with both time and drug having a significant effect ($p<0.001$ and $p=0.0014$ respectively). There is no difference in morphine levels at 20, 30 and 45 minutes between the groups, but higher levels are seen in the codeine animals at 10 minutes ($p<0.001$).

Comparison of M-3-G levels between the two groups shows no significant interaction between drug and time. Time does not have a significant effect but drug does, ($p<0.0001$). At all time points higher levels of M-3-G are seen in the animals treated with codeine, $p<0.001$ at 10, 20 and 30 minutes and $p<0.01$ at 45 minutes. M-6-G is not detected in any of the animals receiving morphine and is detected at low levels in the occasional animal receiving codeine.

DA Adult animals

Table 9 shows the plasma morphine and morphine metabolite levels for DA adult animals following morphine 2mg/kg, and table 10 shows the comparative results for DA adult animals following codeine 10mg/kg. Figure 20 shows a graphical comparison between the two groups of animals for plasma morphine and M-3-G levels over time.

Comparison of morphine levels between the two groups shows no interaction between drug and time. Drug does not have a significant effect but time does ($p=0.0011$). There is no difference in morphine levels between the groups at either time point but there is a difference within both the codeine and morphine animals between their respective morphine levels at 20 and 45 minutes ($p=0.034$ and $p=0.025$ respectively).

Comparing M-3-G levels between the two groups shows no significant interaction between drug and time. There is also no effect of either time or drug either between the two groups or within each separate group. M-6-G is not detected in either the codeine or the morphine animals.

Comparisons across age groups

SD animals

Comparing the SD P10, P21 and adult animals (at 45 minutes) receiving morphine there is no difference between the levels of morphine and M-3-G in the animals at any of the ages.

However, for the animals receiving codeine, morphine levels are not consistent at all ages with P10 and adult animals having higher levels in comparison to the P21 animals ($p<0.05$ and $p<0.001$ respectively), but there is no difference between the P10 and adult animals. Also the M-3-G levels in the animals receiving codeine show a different picture, with levels decreasing with age. There is a difference between the P10 animals and both the P21 and adult animals ($p<0.001$ in each case), but there is no difference between the P21 and adult animals.

The P10 and P21 SD pups receiving codeine are the only animals producing quantifiable levels of M-6-G, though only at low levels that approximate to the level of detection for M-6-G. There is no difference in the levels of M-6-G between these two age groups.

DA animals

In the DA animals comparisons are only possible between the P21 and adult age groups. Adult animals receiving morphine have higher plasma morphine levels than P21 animals ($p=0.014$). The situation is reversed for M-3-G with P21 animals having higher levels than adult animals ($p=0.04$). This pattern of results is also seen in the animals receiving codeine with morphine levels higher in the adult animals ($p=0.011$) and M-3-G levels higher in the P21 animals ($p<0.001$).

Comparisons between rat strains

Once more comparisons are only possible using P21 and adult animals of the two rat strains. For the P21 animals, in both the codeine and morphine groups, there is no difference in morphine levels between the two strains. However, M-3-G levels are higher in the DA strain, in animals in both the codeine and morphine groups ($p<0.001$ in both cases).

The results for adult animals (at 45 minutes) are different. For animals receiving morphine there is no difference between the two strains for either plasma morphine or M-3-G levels. Whereas for the animals receiving codeine there is again no difference between the two strains in morphine levels, but the SD animals have higher M-3-G levels ($p=0.01$).

3.3.3 Plasma morphine and metabolite ratios

Table 11 shows a comparison of the calculated metabolite:morphine and metabolite:metabolite ratios for all the animals studied. Not all ratios could be calculated due to results that were unquantifiable and the fact that M-6-G was not detected in most groups analysed, especially in those groups receiving morphine.

SD animals

The ratios of M-3-G:morphine (M) for SD animals were similar across the age groups following morphine. However, following codeine the ratio varied widely at all ages being highest at P21 and lowest in the adult. In both groups M-3-G was present in much higher concentrations than morphine. However, there is a difference between the drug groups with the ratio being higher in the codeine animals at all ages.

The ratios of M-3-G:M-6-G and M-6-G:M could only be calculated in the SD P10 and P21 animals. The M-3-G:M-6-G ratio fell as age increased but the M-6-G:M ratio increased with increasing age.

DA animals

In the DA animals only the M-3-G:M ratios for the P21 and adult animals could be calculated. The ratios in the codeine group were 1.5-2 times higher than those in the morphine group at each age. Within each drug group the ratio is 5-6 times greater in the P21 animals compared with adult animals.

Comparison between strains

Once more comparisons can only be made using the M-3-G:M ratios in P21 and adult animals. At P21 the ratio is much higher in the DA animals following both codeine (1.5 times greater) and morphine (5.9 times greater). However, in the adult animals following codeine the ratio is marginally higher in the SD animals (1.2 times) whilst following morphine the ratio is slightly higher in the DA animals.

Plasma morphine level versus effect

The correlation between plasma morphine level and the change in mechanical threshold at the time of morphine sampling was calculated. Pearsons correlation was used to calculate a correlation coefficient and a p value, with $p<0.05$ considered significant.

This calculation was only possible in the SD P10 and P21 and the DA P21 animals receiving both codeine and morphine. None of these groups showed an association between plasma morphine levels and effect.

Notes on results tables

When analysing small plasma samples, it was often not possible to quantify the results. If morphine or its metabolites were reliably detected in such a sample the result was recorded as "detected". If they were not detected it was not possible to state with

confidence that they were below the limits of detection due to the same small sample sizes. Thus the result was recorded as "no result" (NR). For plasma samples in which quantification was possible, the levels of morphine, M-3-G and M-6-G are given in ng/ml and in those samples in which they are not detected the result is given as "not detected" (ND).

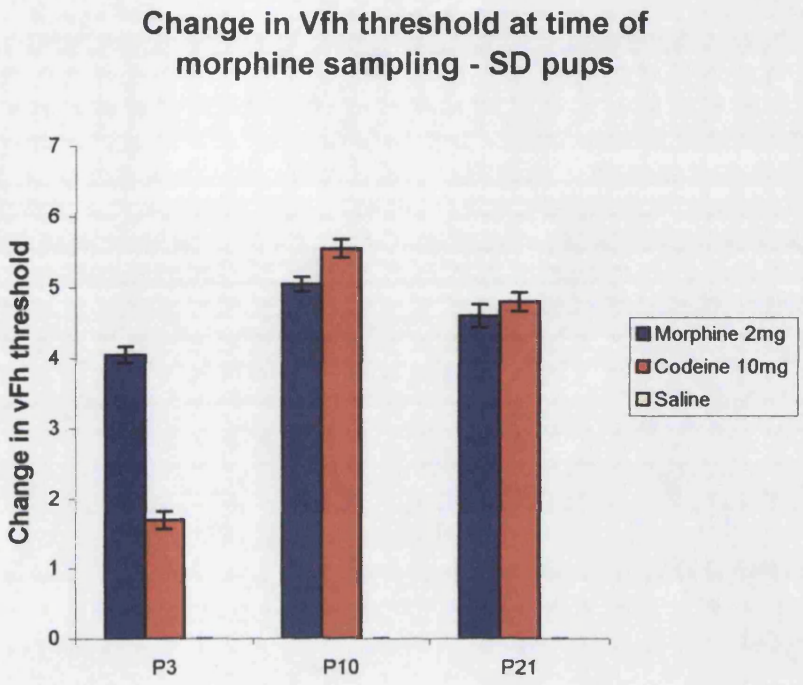

Figure 17. Comparison of the change in vFh threshold required to elicit the flexion withdrawal reflex in SD rat pups following the intra-peritoneal injection of codeine, morphine or saline. Measurements were taken immediately prior to morphine sampling. Displayed are mean and standard error of the mean. ANOVA was used to analyse these data (see text).

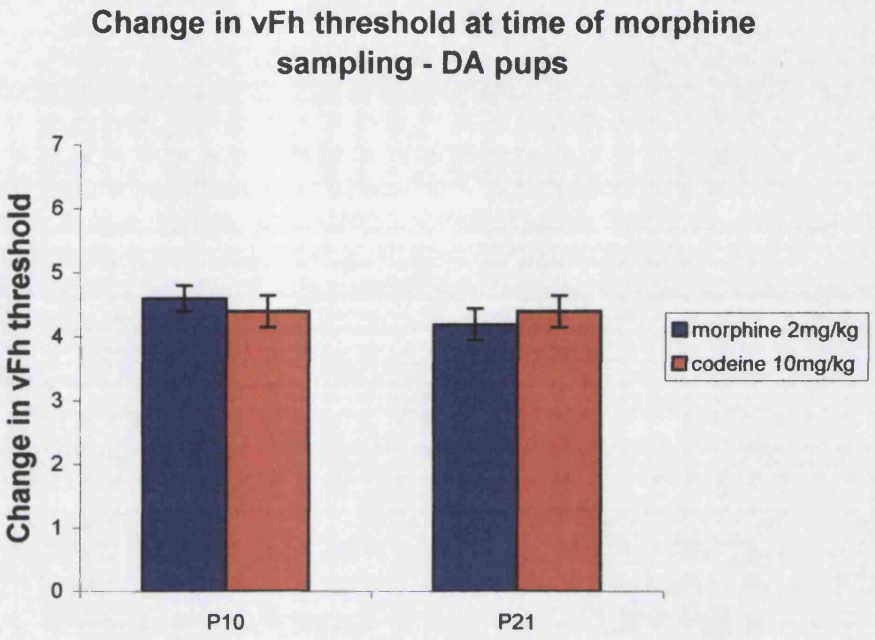

Figure 18. Comparison of the change in vFh threshold required to elicit the flexion withdrawal reflex in DA rat pups following the intra-peritoneal injection of codeine or morphine. Measurements were taken immediately prior to morphine sampling. Displayed are mean and standard error of the mean. ANOVA was used to analyse these data (see text).

A: Codeine 10mg/kg

Animal	Morphine (ng/ml)	M-3-G (ng/ml)	M-6-G (ng/ml)	Effect (vFh +/-)
1	Detected	Detected	NR	1
2	Detected	Detected	NR	2
3	Detected	Detected	NR	2
4	Detected	Detected	NR	1
5	25	2862	NR	3
8	Detected	Detected	NR	3
9	Detected	Detected	Detected	2
10	NR	NR	NR	1
15	Detected	Detected	NR	2
Average				1.9
Standard deviation				0.78

B: Morphine 2mg/kg

Animal	Morphine (ng/ml)	M-3-G (ng/ml)	M-6-G (ng/ml)	Effect (vFh +/-)
4	NR	NR	NR	4
6	Detected	Detected	NR	4
8	NR	Detected	NR	4
9	Detected	Detected	NR	4
10	Detected	Detected	NR	3
12	NR	Detected	NR	4
Average				3.8
Standard deviation				0.41

C: Saline

Animal	Morphine (ng/ml)	M-3-G (ng/ml)	M-6-G (ng/ml)	Effect (vFh +/-)
11	NR	NR	NR	0

Table 2. Plasma morphine and morphine metabolite levels for SD P3 pups. NR = no result.

A: Codeine 10mg/kg

Animal	Morphine (ng/ml)	M-3-G (ng/ml)	M-6-G (ng/ml)	Effect (vFh +/-)
2	Detected	Detected	Detected	6
4	Detected	Detected	Detected	5
5	44	2504	5-15	4
7	Detected	Detected	Detected	6
8	21	2072	5-15	6
9	Detected	Detected	Detected	5
10	27	1966	5-15	6
12	39	2006	5-15	6
Average	32.75	2137	10	5.5
Standard deviation	10.6	248.5	0	0.76

B: Morphine 2mg/kg

Animal	Morphine (ng/ml)	M-3-G (ng/ml)	M-6-G (ng/ml)	Effect (vFh +/-)
2	27	633	ND	5
5	41	765	ND	5
7	NR	NR	NR	5
11	NR	NR	NR	5
12	NR	319	ND	5
13	Detected	Detected	NR	4
14	33	683	ND	5
15	28	717	ND	5
Average	32.25	623.4		4.9
Standard deviation	6.4	176.9		0.35

C: Saline

Animal	Morphine (ng/ml)	M-3-G (ng/ml)	M-6-G (ng/ml)	Effect (vFh +/-)
1	ND	ND	ND	0
4	ND	ND	ND	0

Table 3. Plasma morphine and morphine metabolite levels for SD P10 pups. NR = no result, ND = not detected.

A: Codeine 10mg/kg

Animal	Morphine (ng/ml)	M-3-G (ng/ml)	M-6-G (ng/ml)	Effect (vFh +/-)
2	12	1256	5-15	5
3	13	1244	6	5
4	13	1198	5-15	4
5	5-10	1250	5-15	5
6	5-10	987	5-15	4
7	5-10	1215	5-15	5
10	13	1324	5-15	5
11	5-10	NR	5-15	5
Average	10.1	1210.6	9.5	4.8
Standard deviation	2.8	106.3	1.4	0.46

B: Morphine 2mg/kg

Animal	Morphine (ng/ml)	M-3-G (ng/ml)	M-6-G (ng/ml)	Effect (vFh +/-)
1	34	497	ND	5
2	13	358	ND	5
3	90	430	ND	4
4	15	448	ND	5
5	13	426	ND	4
6	19	619	ND	5
7	17	446	ND	5
8	13	448	ND	4
9	25	103	ND	4
10	40	441	ND	5
Average	27.9	451.6		4.6
Standard deviation	23.8	68.7		0.52

C: Saline

Sample for animal injected with saline haemolysed. Thus not suitable for analysis by HPLC.

Table 4. Plasma morphine and morphine metabolite levels for SD P21 pups. NR = no result, ND = not detected.

A: Codeine 10mg/kg

Animal	Morphine (ng/ml)	M-3-G (ng/ml)	M-6-G (ng/ml)	Effect (vFh +/-)
1	NR	Detected	Detected	4
2	NR	Detected	NR	5
3	NR	Detected	NR	5
4	NR	Detected	Detected	4
5	ND	1256	5-15	4
Average				4.4
Standard deviation				0.58

B: Morphine 2mg/kg

Animal	Morphine (ng/ml)	M-3-G (ng/ml)	M-6-G (ng/ml)	Effect (vFh +/-)
2	23	998	ND	4
3	ND	194	ND	5
4	5-10	880	ND	5
5	ND	306	17	4
Average		594.5		4.5
Standard deviation		403.3		0.58

Table 5. Plasma morphine and morphine metabolite levels for DA P10 pups. NR = no result, ND = not detected.

A: Codeine 10mg/kg

Animal	Morphine (ng/ml)	M-3-G (ng/ml)	M-6-G (ng/ml)	Effect (vFh +/-)
1	16	2432	ND	4
2	12	3027	ND	5
3	14	2789	ND	5
4	21	2415	ND	4
5	12	2719	ND	4
Average	15	2676.4		4.4
Standard deviation	3.7	257.6		0.55

B: Morphine 2mg/kg

Animal	Morphine (ng/ml)	M-3-G (ng/ml)	M-6-G (ng/ml)	Effect (vFh +/-)
1	13	2322	ND	4
2	19	1159	ND	4
3	ND	819	ND	5
4	13	1264	ND	4
5	17	1767	ND	4
Average	15.5	1466.2		4.2
Standard deviation	3	586.7		0.45

Table 6. Plasma morphine and morphine metabolite levels for DA P21 pups. ND = not detected.

A: Morphine ng/ml

Animal Number	10 (min)	20 (min)	30 (min)	45 (min)
1	191		42	
2	58		53	
3	67		483*	
4	57		599*	
5		88		43
6		246*		36
7		78		46
Average	93.25	83	47.5	41.67
Standard deviation	65.32	7.1	7.8	5.13

B: M-3-G ng/ml

Animal Number	10 (min)	20 (min)	30 (min)	45 (min)
1	493		550	
2	471		1018	
3	286		877	
4	952		1110	
5		546		727
6		877		788
7		568		502
Average	550.5	663.7	888.8	672.3
Standard deviation	283.3	158.1	245.3	150.6

C: M-6-G mg/ml

Animal Number	10 (min)	20 (min)	30 (min)	45 (min)
1	ND		ND	
2	ND		ND	
3	ND		ND	
4	ND		ND	
5		ND		ND
6		ND		ND
7		ND		ND
Average				
Standard deviation				

Table 7. Plasma morphine and morphine metabolite levels for SD Adult (P63-70) following morphine 2mg/kg. ND = not detected. * treated as biological outliers.

A: Morphine ng/ml

Animal Number	10 (min)	20 (min)	30 (min)	45 (min)
1			52	36
2		25		
3		143	98	
4	231			60
5	338		88	
6	368		96	
7		71		39
8		44		23
9		62		63
Average	312.3	69	83.5	44.2
Standard deviation	72	45	21.4	16.9

B: M-3-G ng/ml

Animal Number	10 (min)	20 (min)	30 (min)	45 (min)
1			1809	1244
2		1583		
3		1841	1769	
4	1554			1145
5	1798		1558	
6	1825		1800	
7		1816		1488
8		977		1405
9		1812		1818
Average	1725.7	1609.8	1734	1416
Standard deviation	149.3	358.2	118.6	263.3

C: M-6-G mg/ml

Animal Number	10 (min)	20 (min)	30 (min)	45 (min)
1			Not Detected	11
2		13		
3		Not Detected	Not Detected	
4	Not Detected			Not Detected
5	Not Detected		Not Detected	
6	11		Not Detected	
7		Not Detected		Not Detected
8		Not Detected		Not Detected
9		Not Detected		18

Table 8. Plasma morphine and morphine metabolite levels for SD Adult (P63-70) following Codeine 10mg/kg. ND = not detected

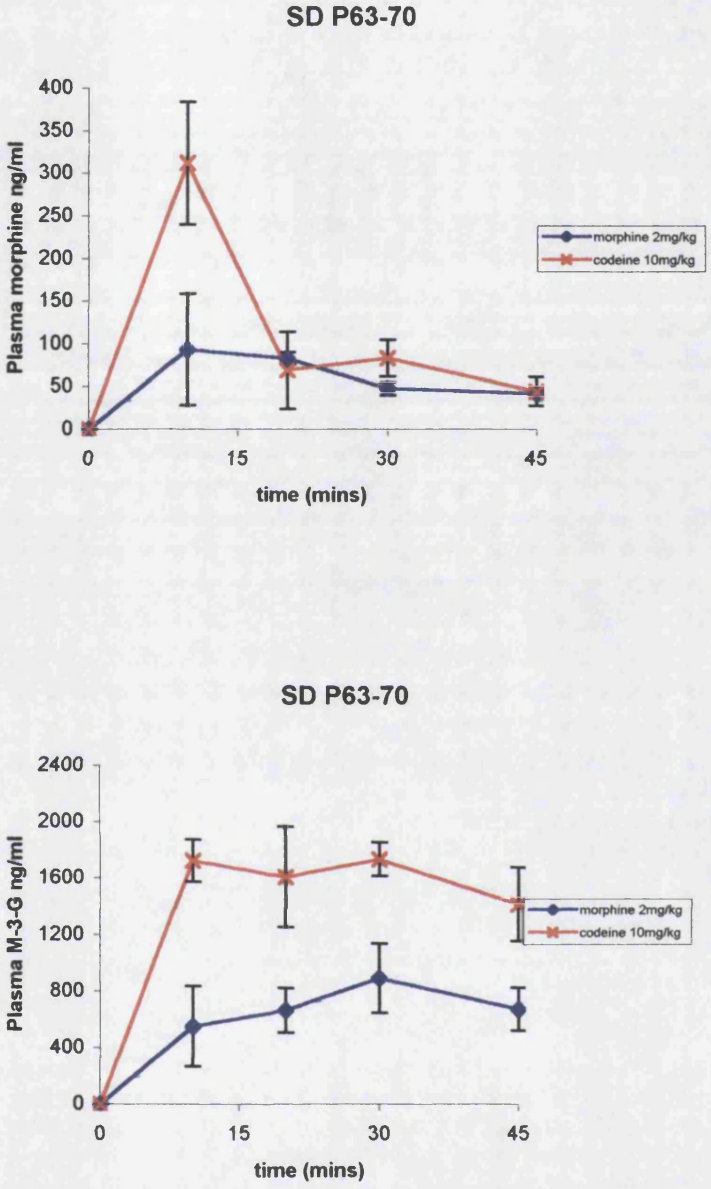

Figure 19. Plasma morphine and morphine metabolite levels for SD Adult (P63-70) following codeine 10mg/kg and morphine 2mg/kg. Displayed are mean and standard deviation.

A: Morphine ng/ml

Animal Number	20 (min)	45 (min)
1	ND	ND
2	57	32
3	65	25
Average	61	28.5
Standard deviation	5.7	5

B: M-3-G ng/ml

Animal Number	20 (min)	45 (min)
1	264	326
2	899	595
3	705	674
Average	622.7	531.7
Standard deviation	325.4	182.4

C: M-6-G ng/ml

Animal Number	20 (min)	45 (min)
1	ND	ND
2	ND	ND
3	ND	ND

Table 9. Plasma morphine and morphine metabolite levels for DA adult (P63-70) following Morphine 2mg/kg. ND = not detected.

A: Morphine ng/ml

Animal Number	20 (min)	45 (min)
1	46	36
2	56	30
3	42	21
Average	48	29
Standard deviation	7.2	7.6

B: M-3-G ng/ml

Animal Number	20 (min)	45 (min)
1	468	507
2	950	1068
3	1152	741
Average	856.7	772
Standard deviation	351.4	281.8

C: M-6-G ng/ml

Animal Number	20 (min)	45 (min)
1	ND	ND
2	ND	ND
3	ND	ND

Table 10. Plasma morphine and morphine metabolite levels for DA Adult (P63-70) following Codeine 10mg/kg. ND = not detected.

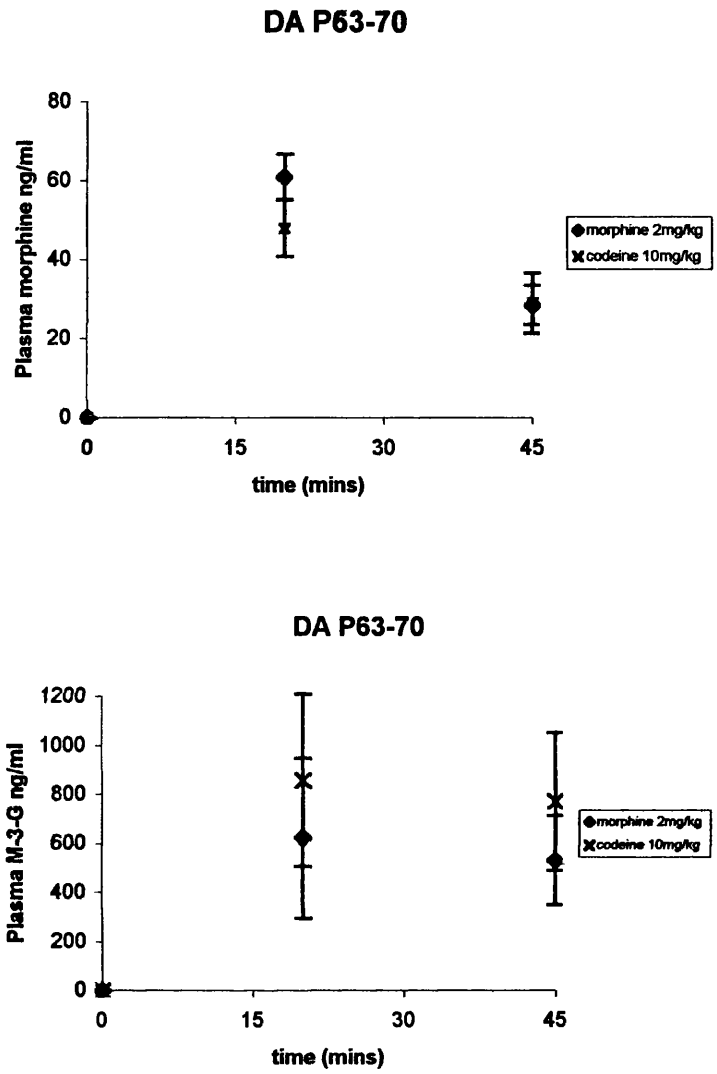

Figure 20. Plasma morphine and morphine metabolite levels for DA Adult (P63-70) following codeine 10mg/kg and morphine 2mg/kg. Displayed are mean and standard deviation.

A: SD animals

		P3	P10	P21	Adult (45 minutes)
Codeine	M-3-G:M-6-G		213.7:1	127:1	
	M-3-G:M		65.3:1	121:1	32:1
	M-6-G:M		0.3:1	0.94:1	
Morphine	M-3-G:M-6-G				
	M-3-G:M		19.3:1	16.1:1	16.1:1
	M-6-G:M				

B: DA animals

		P10	P21	Adult (45 minutes)
Codeine	M-3-G:M-6-G			
	M-3-G:M		178:1	26:1
	M-6-G:M			
Morphine	M-3-G:M-6-G			
	M-3-G:M		94.6:1	18.6:1
	M-6-G:M			

Table 11. Comparison of the ratios of plasma morphine and its metabolites in SD and DA animals at all the ages studied. Figures are only given where a ratio can be calculated.

3.4 Discussion

The measurement of plasma morphine and metabolite levels, following either codeine or morphine administration, has not been previously undertaken in the developing rat. Plasma levels were used to provide an indirect measure of enzyme activity and hence morphine production in an attempt to explain the differences in behavioural effects seen between age groups and strains.

3.4.1 Morphine sampling effects

In each animal the baseline mechanical threshold was measured prior to injection of the opioid and the change in threshold was measured immediately prior to morphine sampling. The results from these current experiments mirrored the results from the behavioural work, thereby emphasizing the reproducibility of the effects seen and showing once again that there is developmental regulation of the efficacy of codeine. Occasional small differences were seen and can probably be attributed to the fact that all the tests were done at a fixed time whereas during the behavioural work there was a small amount of variation in the time to peak effect.

3.4.2 Plasma morphine and morphine metabolite levels

SD animals

Results from the behavioural studies show that both codeine and morphine are effective at P10, P21 and P63-70. Sampling results show that at each of these ages codeine is metabolised to morphine and morphine metabolites and the levels are broadly similar to those animals given morphine. Whilst great care must be taken when extrapolating plasma levels to effect, these results do suggest that the morphine metabolised from a dose of codeine is playing a significant part in the analgesic effect produced.

Although P3 animals given codeine demonstrated a much smaller behavioural response, morphine and M-3-G were also detected in these animals. This is not a totally surprising result since, at this age, there is some, though comparatively much reduced, O-

demethylase activity.(Matsubara T et al 1986) Though with no quantifiable results, no comparison can be made of the morphine and metabolite levels with those in older animals treated with codeine and animals treated with morphine at all ages.

At P10, P21 and P63-70 morphine and M-3-G levels at 45 minutes varied with age following codeine but not morphine. A possible explanation for this is the differences in O-demethylase activity between the ages. However, this cannot completely explain the differences seen as enzyme activity is similar at P10 and P21 and lower in the adults which does not correlate with the variations in plasma levels.(Matsubara T et al 1986) Activity of enzymes is also dependent on membrane phospholipid concentration which varies with age in the rat.(Kapitulnik J et al 1986) Differences in pharmacokinetics between the ages may also have an effect on the results.

The overall trends of plasma levels of morphine and its metabolites in the adult animals are in agreement with results seen in previous studies. In the adult animals morphine levels following administration of morphine appeared constant over time. However, the first sample was taken at ten minutes and previous work in male SD rats suggests that peak plasma levels following intra-peritoneal injection occur before this, with levels then declining. Levels were lower than those seen after 4mg/kg but higher than after 1mg/kg.(Zheng M et al 1998; Hasselstrom J et al 1996) Following codeine, peak morphine levels are seen at ten minutes which drop away soon after. This is in line with previous work that shows peak levels of morphine following codeine in adult male SD rats to be at around 2-6 minutes following intravenous administration.(Shah J et al 1991) It is to be expected that intra-peritoneal injection would extend this time to allow for absorption. Levels were lower in previous work but doses of 1-4mg/kg were used compared to 10mg/kg in these experiments.

The quantitative differences in the plasma levels between these current experiments and the previous work may be due to different doses of drug used, different routes of administration (in the case of codeine) and the use of animals of a different sex, as rats are known to show sex related differences in drug metabolism(Kamataki T et al 1983; Kamataki T et al 1982; Kato R 1974) For example, O-dealkylase activity in SD rats increases with age in both sexes up to P25-30, in the male rat these levels of activity

then remain into adult life but in the female the activity declines to around 25-50% of the male levels by P40 and remain there into adult life.(Matsubara T et al 1986)

Following both codeine and morphine, M-3-G is the major metabolite seen in all animals. This agrees with previous work in adult male SD animals treated with morphine.(Zheng M et al 1998; Hasselstrom J et al 1996) It is present at very high levels in comparison with morphine. M-3-G has no analgesic effect but has been suggested to be antagonistic to the actions of morphine.(Gong QL et al 1992; Smith MT et al 1990) However, these results would question this theory due to the fact that in spite of the high levels of M-3-G seen following both codeine and morphine, significant effects are still seen.

In the current experiments M-6-G was only detected occasionally at low levels supporting the previous conclisions that in rats the formation of M-6-G is only a minor route of the metabolism of morphine and as such M-6-G, despite its known analgesic potency, is unlikely to have as a significant effect on the analgesic efficacy of codeine and morphine in rats as it does in humans. (Zheng M et al 1998; Hasselstrom J et al 1996; Coughtrie MWH et al 1989; Rane A et al 1985)

DA animals

Morphine and M-3-G, but not M-6-G, were detected at all ages tested following both codeine and morphine administration. The role of the DA rat as a PM of codeine has been questioned and the CYP2D1 deficiency is thought to be relative and can be dependent on age and substrate concentration. Also the handling of some compounds in rats may be different compared to humans and involve more than one cytochrome P450.(Barham HM et al 1994; Vincent-Viry M et al 1988; Boobis AR et al 1986; Khan GC et al 1985) There are 5 CYP2D isosymes and their relative activity and contribution to codeine metabolism during different stages of development are not known.(Kawashima H et al 1995; Matsunaga E et al 1990; Gonzalez FJ et al 1987) Whether the activity of CYP2D1 varies at these ages in the DA rat is also unknown.

As with the SD rats M-3-G is the major metabolite seen with no M-6-G detected in any DA animal. In the P21 animals DA rats had higher levels of M-3-G than the SD animals, though in adult animals levels of M-3-G were similar between the strains following morphine and higher in the SD animals following codeine. This suggests that the overall metabolism of morphine and the production of M-3-G varies between the strains and may show a different developmental pattern within each strain. This may point to other cytochrome P450 enzymes having effects, which vary between the two strains or highlight different levels of CYP2D1 activity between the strains.

Once again the metabolism of codeine to morphine in these DA animals and the broadly similar plasma levels compared with the DA animals treated with morphine, combined with the effects seen in the behavioural work, suggests that the metabolism to morphine is important for the analgesic effect seen in DA rats following codeine.

This, however, gives one result that is difficult to explain. Adult DA rats given codeine showed no analgesic effect, as seen in previous studies, but plasma sampling showed significant amounts of morphine and M-3-G are produced, which is in contrast to previous work done on rat liver microsomes in vivo.(Cleary J et al 1994; Mikus G et al 1991) The morphine levels are similar to those seen in adult DA rats treated with morphine and adult SD rats treated with both codeine and morphine, all of which showed a significant effect. A potential reason is that the overall metabolism is reduced in these adult DA animals given codeine. In the P21 DA animals the M-3-G:M ratios are higher than those seen in the P21 SD animals following both codeine and morphine. In the adult animals the same picture is seen following morphine but following codeine the ratio is higher in the SD animals. Ratios of morphine and its metabolites are used as a measure of the overall pharmacological action of morphine and thus this decreased ratio in adult DA rats given codeine may be a marker of decreased overall effect.(McQuay H et al 1997)

3.4.3 Plasma morphine and metabolite ratios

The M-3-G:M ratios in both SD and DA animals followed the same trends across the age groups as seen for the plasma levels, being similar following morphine but showing

some variation following codeine. M-3-G formation was higher following codeine compared with morphine at all ages and was also higher in DA rats than SD rat, except in the DA adults given codeine as previously mentioned. This again suggests overall differences in metabolism of codeine between the age groups and strains and may again be potentially explained by differences in enzyme activity or variations in pharmacokinetics. However, levels could only be measured at one time point, which may not give an accurate overall picture of metabolism.

The ratios at all ages are higher than those seen in previous work in adult male SD rats following morphine.(Zheng M et al 1998; Hasselstrom J et al 1996) Again differences in metabolism could be explained by different doses and possible sex differences. The ratios are also higher than those seen in humans following intravenous administration but lower than those seen after oral administration.(Faura CC et al 1998)These differences could potentially be attributed to species differences in metabolism and different routes of administration.

The M-3-G:M-6-G could only be calculated following codeine in the P10 and P21 animals and decreased with age, whereas in humans the ratio increases with age.(Faura CC et al 1998) Though again the significance of this is not clear due to the very low levels of M-6-G produced at all ages and the probable negligible effect this metabolite plays in the efficacy of codeine and morphine in rats.

3.4.4 Correlation of plasma levels with effect

No association was found between morphine levels and effect following codeine or morphine in any of these groups. This is in agreement with previous work in humans that has shown no association between plasma morphine levels and analgesic efficacy.(Kart T et al 1997; Millar AJW et al 1987; Lynn AM et al 1984; Nahata MC et al 1984)

The explanation for this is that it is not plasma levels of morphine that are important for efficacy but binding to opioid receptors and transduction. Thus, other factors maybe of more relevance, such as the CSF morphine and metabolite concentrations, metabolism

occurring within the CNS, concentrations locally to receptors, ease of agonist/receptor binding and receptor density. It is known that development affects some of these factors, for example the relative densities of the different opioid receptors changes during neonatal life.(Rahman W et al 1998; Attali B et al 1990; Leslie FM et al 1982) However, little is known how potential variations in each of these factors during development may affect analgesic efficacy.

In adult rats, following the administration of morphine, morphine has been shown to be present in the CNS, as have codeine and morphine following administration of codeine.(Barjavel M et al 1994; Dahlstrom B et al 1976) Codeine penetrates the blood brain barrier more rapidly than morphine and metabolism of both codeine and morphine can occur locally in the CNS.(McQuay H et al 1997; Chen ZR et al 1990; Oldendorf WH et al 1972) This adds weight to the argument that plasma levels are unrelated to effect and may also be used to explain why peak plasma levels occur rapidly following administration but peak effect occurs later when plasma levels have declined.

3.4.5 Summary

Following the administration of codeine to rats of both strains morphine is detected in the plasma at all ages. The levels seen vary across the ages, which may be due to developmental differences in enzyme activity and opioid pharmacology, but are broadly similar to those seen following morphine at each age. Although we have shown there is no correlation between plasma levels of morphine and effect, this suggests that morphine probably does play a significant part in the analgesic effect seen from a dose of codeine.

The results also show that the use of the DA strain as a model of a human PM must be done with caution. The ability to metabolise codeine to morphine and behavioural effects are present in the developing animal. Whether this is due to relative differences of CYP2D1 activity with age and/or the involvement of other cytochromes P450, the activities of which may also vary with age, is unknown. More work in this area is needed to clarify this.

However, some surprising results were seen for which there is no satisfactory explanation at present. In adult DA animals following codeine, morphine and M-3-G were produced at levels broadly similar to the other animals but a comparatively decreased effect was observed in these animals during the behavioural work. Technical problems, decreased overall metabolism, as calculated by the M-3-G:M ratio, or other factors, such as developmental and genetic differences in codeine transport across the BBB and interaction with opioid receptors, may also play a part. Though none of these reasons gives a wholly credible explanation and again more work is required to explain these results.

In the younger animals blood could only be taken at one time point and it is difficult to draw precise conclusions about overall enzyme activity and pharmacokinetics on limited information. Further work could involve repeating these experiments at each age group with blood being taken at a number of different time points, as was done with the adult animals, though in the younger animals there would only be one sample per animal. Also measuring levels from CSF or brain homogonates could possibly give a clearer picture with regard to the extent of metabolism in relation to effect.

Measuring plasma morphine levels was an indirect measure of enzyme activity. A more direct method would be to use liver microsomes in vivo from animals at each age group and measure enzyme activity directly as has been done in studies in humans.(Jacqz-Aigrain E et al 1992)

CHAPTER 4. CLINICAL STUDY

4.1 Introduction

Codeine is still widely prescribed for postoperative pain relief and is frequently recommended for paediatric use.(Dolhery C 1999; Prevention and Control of Pain in Children 1997; Schechter NL et al 1993) The reputedly lower incidence of opioid - related side effects has made codeine popular for the younger age groups including neonates and especially in situations where airway management and neurological assessment are critical.(Semple D et al 1999; Husband AD et al 1996a; Stoneham MD et al 1995; Hatch DJ et al 1995; Lloyd-Thomas AR 1990) However, animal and adult human laboratory experimental studies have shown significant variability in both the pharmacokinetics and pharmacodynamics of codeine and that the analgesic effect of codeine is dependent on its metabolism to morphine.(Wilder-Smith CH et al 1998; Eckhardt K et al 1997; Cleary J et al 1994; Mikus G et al 1991; Yue QY et al 1991; Chen ZR et al 1991; Sindrup SH et al 1990)

Both adult and paediatric clinical studies have demonstrated that although the incidence of side effects may be low, the overall efficacy of codeine is also low and analgesia may be inadequate for postoperative pain in some circumstances. (McEwan A et al 2000; Semple D et al 1999; Quiding H et al 1993; Persson K et al 1992)

One potential cause of this variability and low efficacy is genetic polymorphism. Over 50 different genetic variants are known to exist for CYP2D6, the enzyme that converts codeine to morphine, which leads to a wide spectrum of metabolic capabilities within populations.(Marez D et al 1997; Daly AK et al 1996) There have been very few clinical studies investigating the analgesic efficacy of codeine in which genetic variation is taken into consideration in adults, and none in paediatric groups. In the only study of postoperative patients to consider this factor, the analgesic efficacy of codeine was found to be low but overall there was no difference in efficacy between the two phenotypes (EM and PM), however, the numbers of PMs was very small.(Poulsen L et al 1998)

The estimated frequency of PMs in the UK population is 9%, however, there is wide variation among ethnic groups, being about 1% in Arabs, 7% in Caucasians and 30% in Hong Kong Chinese.(Sachse C et al 1997; Alvan G et al 1990; Zhou H-H et al 1989) Thus are we giving a drug to 9% of the UK population that has no analgesic effect and a variable effect in the rest of the population? Is the perceived lack of side effects really related to a lack of morphine produced and hence also a lack of analgesic effect? Should we be using analgesic agents with a more reliable effect such as morphine and would using morphine give an acceptable side effect profile?

The aim of this clinical study is to use a peri-operative model in children to try and answer these questions. Primarily to look at the overall analgesic efficacy and side-effect profile of codeine compared with morphine but also to relate these outcomes in terms of the genotypes of the children for the CYP2D6 enzyme and the plasma levels of morphine and it glucuronide metabolites.

4.1.1 Operative model

Adenotonsillectomy was chosen as the operative model as it is still one of the most common surgical procedures performed in paediatric medicine. It can result in significant postoperative pain and distress and an effective analgesic regimen is essential to prevent impaired swallowing, which may lead to dehydration and infection of the tonsillar bed.(Warnock FF et al 1998; Husband AD et al 1996b) Ideally the analgesic technique used should be able to provide adequate pain relief, preserve laryngeal reflexes, avoid excessive sedation and not cause vomiting.

None of the currently employed analgesic regimens provides all these features. Local anaesthetic infiltration is considered to be inadequate without other analgesics and is not universally employed.(Stuart JC et al 1994) NSAIDs are not sufficient for all patients and maybe associated with an increased risk of bleeding.(Splinter WM et al 1996; Gunter JB et al 1995) Mouth rinses, gargles and lozenges are also inadequate when used alone. Commonly used opioids, such as morphine, may lead to excessive sedation, and thus potentially to airway obstruction and pulmonary aspiration, and a high incidence of postoperative nausea and vomiting.

Thus codeine, due to its perceived benefits of good analgesia and low incidence of opioid related side-effects, is often used for peri-operative analgesia during adenotonsillectomy in combination with simple analgesics such as NSAIDs and paracetamol with further simple analgesics and opioid, if required, given for a number of days postoperatively.

4.1.2 Assessment of analgesic efficacy

How best to assess analgesic efficacy has been and still is the subject of intense debate. This is due to the fact that the perception of pain is a subjective experience and that there is no single parameter or group of parameters that show sufficient specificity and sensitivity for the measurement of pain.

In children these problems are compounded by the age related differences in physiology, behavioural responses, cognitive abilities and psychological development seen during development. In addition sociocultural and environmental factors will also influence the assessment of pain. The assessment of pain in children involves a complex holistic process taking all these factors into consideration.(Morton NS 1977) The pain tools and aids that are often used in research for data collection and comparisons are only really aids to an overall process of pain management of each child.

This leads to significant problems when deciding how best to compare the analgesic efficacy of different treatment regimens during clinical trials. There is no single technique that does not leave the interpretation of the results open to debate. This has led to the use of different techniques among investigators with no overall consensus. For this study two techniques of assessment were used to try and overcome these problems and also to see if there was agreement between these techniques.

Time to first analgesia

This method of data collection and analysis is commonly used in pain research as in many other clinical fields and is based on using time to event (survival) data.(Altman DG et al 1998) The patient is regularly assessed after the analgesic intervention, in this case the administration of the opioid to be studied, to see at what time point further analgesia is required. A suitable end point is identified based on the characteristics of the treatment regimens being studied and the patient is said to have "survived", if no further analgesia is required during this time, or "died" at the time analgesia was given. This data can then be analysed by the use of survival analysis, such as the Kaplan-Meyer method, to draw survival curves and draw comparisons between the curves for the different treatment regimens to give a significance value.

The decision to administer further analgesia can be made by the patient themselves if the analgesic regimen allows for this e.g. patient controlled analgesia or based on clinical grounds, assessed by the medical staff caring for the patient, or by using a fixed point on a pain assessment tool or aid, at or above which the child is deemed to require further analgesia. Each method is open to criticism, the subjective nature of pain perception may be a source of error when comparing patients using self-administration and the use of a fixed point on a pain tool relies on the accuracy of that pain tool which, for all the reasons discussed, cannot be considered to be an exactly reliable technique between patients. Assessment on clinical grounds can be criticised as it will be influenced by the opinions of the carer, but as this is the endpoint most often used in the everyday care of patients it was decided to use this as the basis for the administration of analgesia during this current study.

Two survival times were chosen for analysis. Longer than four hours after opioid injection to encompass the duration of action of both intra-muscular codeine and morphine(British National Formulary 1999) and shorter than two hours to highlight those patients who needed further analgesia in the early postoperative period.

Pain scoring

As discussed there is no easily administered, widely accepted technique for pain assessment in children. Though this is an area of ongoing research, and there is now a vast literature concerning pain assessment and scoring in children.(Franck LS et al 2000) Guidelines and recommendations for pain assessment have been produced by professional bodies but there is little consensus on use and no single tool has gained wide acceptance.(Clinical Guidelines for the Recognition and Assessment of Acute Pain in Children. 1999) The choice of tool depends on age, the population and setting in which it is to be used, and the preferences of those involved.

Assessment techniques can be classified as self-reports, behavioural observations or physiological measures. Some assessments are multi-dimensional and incorporate more than one measure e.g. behavioural and physiological, or asses different aspects of the pain experience e.g. intensity, location, pattern, context and meaning.

Self-report pain assessment

Due to the fact that pain is a subjective experience self-report methods are considered to be the gold standard of pain assessment. By 3 to 4 years of age cognitive development is sufficient to allow reporting of the degree of pain and so self-reporting tools can be used from this age onwards.(Harbeck C et al 1992) A number of different tools have been developed ranging from simple numerical and descriptive scales to graphical scales in which images such as faces and poker chips illustrate increasing degrees of pain.

A scale employing cartoon faces was developed and verified by Whalley and Wong and it, or numerous variations, has been widely used since.(Wong D et al 1986) A study comparing this faces scale with five other tools showed that children aged 3 to18 years preferred the faces to other scales although there was no difference between the scales in terms of validity or reliability(Wong D et al 1988) A variation of the Whalley and Wong pain score was already in use on the ward caring for the children to be enrolled in this study and the nursing staff were all experienced in its use. Thus it was decided to retain this self-report score for the current clinical trial.

Behavioural pain assessment

Pain assessment tools that measure pain related behaviours can be used when child self-report cannot be obtained or as a supplement to self-report measures.(McGrath PJ 1998) However, there are problems associated with behavioural scores. Behaviours change with age and can be modified by underlying factors such as mood, anxiety, distress, somnolence and many drug treatments. It has been shown that health care workers consistently underestimate children's pain compared with children's own self-reports.(Romsing J et al 1996; LaMontagne LL et al 1991)

Again a large number of behavioural pain scores have been designed and validated but none has been shown to be suitable in all situations and age groups. The choice of score must be related to the age of the children and to the situation for which it is to be used. Often these scores measure a global behavioural distress at a particular situation and are not specific to pain.

During this study is was decided to record a behavioural pain score to provide a comparison with the self-report score. However, the choice of which particular behavioural pain score to use was difficult. No single score has been validated at the range of ages to be studied for postoperative pain. Two scores were considered. The Objective Pain Scale, which can be used at all ages, but parts of the tool were only validated for 13-18 year olds.(Broadman LM et al 1988) and the Children's Hospital of Eastern Ontario Pain Scale (CHEOPS) which has been validated for postoperative pain and for use in 1-7 year olds.(McGrath PJ et al 1985) Neither score full-filled all the requirements but it was felt that the CHEOPS score was the most appropriate for the current trial.

Pain at rest vs pain on movement

It has been observed from both experimental work and in the clinical situation that pain at rest is sensitive to opioids whereas pain on movement does not respond well to opioids. (Cooper BY et al 1986; Eriksson MBE et al 1982; Beecher HK 1957) Thus during this clinical trial pain scores were recorded at rest and on swallowing, to see if

there was any difference in the pain relief produced by the opioids in these two situations.

4.2.3 Measurement of morphine and its metabolite levels

A blood sample from each patient, taken after the injection of the opioid, allowed measurement of plasma morphine and morphine glucuronide metabolite levels. Little is known about the pharmacokinetics of codeine following intramuscular (i.m.) injection of codeine in children. However, a recent paper comparing rectal and i.m. codeine given to children during neurosurgery showed that the peak morphine levels following i.m. injection of codeine occurred between 30 minutes and two hours after the injection.(McEwan A et al 2000) Thus, as only one blood sample was to be taken off each patient it was decided to take the sample at one hour and combine this with the initial pain scoring in the recovery room.

Measurement of plasma morphine was performed by Innes Kastner and Jonathan De-lima at the Institute of Child Health, London using the same HPLC method described in Chapter 3.

4.2.4 Genotyping the patients

Blood sampling also allowed for DNA extraction, and thus each patients genotype was also investigated. This work was undertaken by Benjamin Fletcher and Dr Mark Thomas of the Molecular Biology Department at University College London.

DNA extraction

DNA was extracted using the phenol-choloroform method. Buffer is added to the whole blood and proteinase K is then added to the sample to digest the protein overnight. Next phenol-choloroform is the added in a 1:1 ratio, which denatures the protein, and the sample spun in the centrifuge. The aqueous layer, which contains the DNA, is pippeted off and placed in a fresh sample tube with salt and chloroform and then re-spun. The

aqueous layer is again transferred to a fresh tube containing isoproponol. The sample is then frozen and the salt and isoproponol cause the DNA to precipitate out. The sample is then cleaned with ethanol, dried and buffer added.

Polymerase chain reaction (PCR)

PCR is used to cut areas of the DNA into sections of around 20-30 base pairs specific to the area of interest. The DNA is mixed with primers specific for the gene to be studied, buffer, magnesium, Taq enzyme and nucleotide building blocks (dNTPs). The mixture is heated to 95°C to denature the DNA to give single strand DNA. It is then cooled to the optimum temperature to allow the primers to anneal to the DNA and then reheated to the specific temperature for the Taq enzyme to work, which uses the dNTPs to add nucleotides to the primers and split the DNA to give the area of interest. This process is amplified to give lots of copies of the target DNA.

With CYP2D6 the situation is more complicated due to the presence close by of two pseudogenes CYP2D8P and CYP2D7 which are inactive but share >90% sequence identity. Thus a two stage PCR is required to first split off a section specific to the CYP2D6 gene. This product is then diluted and used as a template for a second PCR, which produces a 5 PCR product multiplex. Each one of these products is labelled fluoroscopically with a dye that can be recognised by the analyser.

Detection

These PCR products are then digested overnight with restriction enzymes, which allow discrimination of the presence or absence of particular poymorphisms. The digested PCR is added to size standard and buffer and then run on an ABI – 377 analyser, which detects the products present. This data is analysed electronically with the products identified as a series of peaks compared to the known peaks produced by the size standard.

Polymorphisms detected

To date, over 50 variants for the CYP2D6 gene have been identified and classified.(Marez D et al 1997; Daly AK et al 1996) Most are rare with 87% of genotypes being accounted for by five different variants in the populations studied.(Sachse C et al 1998; Marez D et al 1997) It was impractical to look for all theses different variants in each sample of DNA, thus the most common polymorphisms causing alteration in enzyme activity were targeted. These are shown below: (A=adenine, G=guanine, t=thymine and C=cytosine. **Bold** type is used to show the changes from the *1 polymorphism)

Gene position	100	1023	1846	2549	2615	2850
Polymorphism:						
CYP2D6*1	C	C	G	A	-	C
CYP2D6*2	C	C	G	A	-	**T**
CYP2D6*3	C	C	G	**deletion**	-	C
CYP2D6*4	**T**	C	**A**	A	-	C
CYP2D6*5		**TOTAL**	**GENE**	**DELETION**		
CYP2D6*9	C	C	G	A	**3 base pair deletion**	C
CYP2D6*10	**T**	C	G	A	-	C
CYP2D6*17	C	**T**	G	A	-	**T**

CYP2D6*1 is considered the normal/wild type gene and has normal activity. *3, *4, *5 and *9 have no activity and account for over 90% of PMs, whilst *2, *10 and *17 have reduced activity. If a person is homozygous for any of these genes the activity will be absent or reduced. If a person is heterozygous the activity will be reduced, the amount of reduction will depend on the polymorphism present. Genotyping looked for all the polymorphisms apart from *1 so if none of the polmorphisms were found the sample was considered to be from a patient with the normal gene. Thus there is a chance that a PM patient with another polymorphism may be considered an EM (false negatives), though as these polymorphisms are rare the estimated risk of this occurrence is very small.

Aims of clinical study

1. To assess the analgesic efficacy of codeine and morphine for children undergoing adenotonsillectomy and to compare this with plasma morphine levels and the genotype of the patients.

2. To assess the incidence of side effects following codeine or morphine in this same group of patients.

Hypotheses

1. Patients with the PM phenotype will produce little or no morphine from a dose of codeine compared with the EM phenotype.

2. Patients with the PM phenotype will receive little or no analgesic effect from a dose of codeine.

3. There will be no difference in the incidence of side effects between the two treatment groups.

4.2 METHODS

The study was carried out at The Royal National Throat, Nose and Ear Hospital, Grays Inn Road, London. The local ethical committee approved the study and parental consent was sought for each child prior to enrolment.

Children aged from 3 to12 years, ASA 1 and 2, undergoing tonsillectomy +/- adenoidectomy were recruited. Topical local anaesthesia, to facilitate intravenous cannulation, was given prior to anaesthesia. No other premedication was given. Anaesthesia was induced by the intravenous route, using propofol 2-5 mg/kg or the gaseous route, using oxygen, nitrous oxide and sevoflurane. Once adequate anaesthesia had been established an appropriate size laryngeal mask was inserted into the airway. Anaesthesia was then maintained with oxygen, nitrous oxide and sevoflurane and with the patient breathing spontaneously. Intravenous fluid, Hartmans solution at 10ml/kg, was given throughout the procedure and continued into the postoperative period at a maintenance dose appropriate to the individual child. At the end of the procedure the patient was allowed to breathe 100% oxygen and the laryngeal mask removed once airway reflexes had returned.

All procedures were performed using electrocautery for tonsillar disection and haemostasis. The Anaesthetist and Surgeon present varied depending on which day and on which operating list the procedure was performed.

The patients were randomised into two groups and received an i.m. analgesic injection once anaesthesia had been induced. This was either:

1. Group C - codeine 1.5mg/kg
2. Group M - morphine 0.15mg/kg

Each patient also received diclofenac 1 - 1.5 mg/kg per rectum at the same time.

Patients were kept in the recovery area until they were awake and comfortable and able to return to the ward. Pain scoring was performed in the postoperative period, both in the recovery room or on the ward by the nurse responsible for the care of the patient.

Blood was taken on two occasions via a cannula inserted as part of the anaesthetic technique for the procedure. This was used for:

1. 1-2ml – one hour post injection of opioid. These samples were centrifuged at 3000 rpm for 10 minutes within 15 minutes of being taken. The plasma was then pippeted off and stored prior to processing at -70°C. The plasma was used for the measurement of plasma morphine levels by High Performance Liquid Chromatography. This was performed at the Institute of Child Health by Innes Kastner and Dr Jonathan De-Lima, as described in Section 3.2.4.

2. 2-4ml – after induction, to genotype the patient. This was performed by the Molecular Biology Department at University College London by Benjamin Fletcher and Dr Mark Thomas. The whole blood was stored at -70°C until a batch large enough for processing had been collected.

Further analgesia was given either as intravenous morphine (20mcg/kg bolus), oramorph (0.1mg/kg) or as per rectum or oral paracetamol (20mg/kg) or diclofenac (1mg/kg). The need for extra analgesia was assessed on clinical grounds by the anaesthetist and/or the nurse looking after the patient. Ondansatron 0.1-0.2mg/kg was also used as an anti-emetic if required.

Adverse effects, complications and the incidence of known drug side effects were also recorded. The patient was closely monitored until discharge from the hospital. All staff involved in the procedure and the care of the child were blinded as to which drug had been given to the patient. As the co-ordinator of the project I remained unblinded and as such consented each patient, organised the randomisation, collected the blood samples and prepared the study drug to be given for each case. I took no part in the assessment or collection of data from the patients or administration of analgesic drugs.

Exclusion Criteria

Patients were not included in the study if they were:

1. American Society of Anaesthesiologists (ASA) classification of 3 or above (Kohn RLJ 1985) (table 12)
2. Taking medication that interfered with codeine or morphine metabolism
3. On other analgesic medication
4. Unable to take non steroidal anti-inflammatory drugs
5. Known to have had an adverse reaction to any of the medications to be used

Primary Outcome Measures

- Pain Scores

Two methods of pain scoring were used, a self reporting pain tool (Wong D et al 1986)(figure 21) and the CHEOPS observational pain score (McGrath PJ et al 1985)(table 13), which have been verified in this age group. The children were introduced to the self reporting pain scale at the preoperative visit and shown how to use it. All nursing staff involved in the care of children enrolled in the study were taught how to use the CHEOPS pain score. Scores were recorded in the postoperative period once the patient had roused in the recovery area and at 1, 2, 3, and 4 hours after the end of the procedure. The scores were taken at each time in two situations:

  - At rest
  - On swallowing

- Time to first dose of rescue analgesia.

- Plasma morphine levels – at 1 hour after the injection of the opioid.

- Genotype of the patient.

Secondary Outcome Measures

1. Time to first swallowing of fluid.
2. Length of hospital admission.
3. Respiratory depression – measured by observing the respiratory rate over 1 minute.
4. Sedation score (table 14)
5. Incidence of nausea and vomiting - by counting the number of episodes in set time periods i.e. 0-1 hours, 1-2 hours, 2-4 hours, 4-8 hours and >8 hours after the end of the procedure.
6. Incidence of other complications and adverse events e.g. haemorrhage, delayed discharge etc.

Analysis

Randomisation:

Enrolled patients were randomised into either group M or group C using a standard simple randomisation to ensure equal numbers in both groups e.g. random permuted blocks using random numbers tables.(Altman DG 1991)

Power calculation:

It was not possible to predict preoperatively which patients were PMs and which were EMs. On average 9 -10 % of the UK population are PMs(Ctytochrome P450, subfamily IID; CYP2D 1999), this would suggest that approximately 200 patients are required to have 10 patients in each of the PM groups.

When looking for a difference of 2 standard deviations between two groups, using an alpha level of 0.05 (2 sided) and a statistical power of 80%, the number needed in each group is 8. The difference in plasma morphine levels between was expected to be greater than this since for PMs after codeine dosing, in previous work, the plasma morphine levels were undetectable or significantly lower.(Yue QY et al 1991; Chen ZR

et al 1991; Sindrup SH et al 1990) It was also expected that there will be a large difference in pain scores between the PMs given codeine and the other groups. The aim was to recruit around 200 patients giving approximately 10 patients in each of the codeine and morphine PM groups with the data being analysed as the study progressed and the numbers required adjusted accordingly.

Comparisons:

Comparisons between the two groups were made with both groups treated in their entirety or subdivided into EMs and PMs i.e.

	PM	EM	All the group
Codeine	A	B	L
Morphine	C	D	K

Plasma morphine levels:

- A vs B
- A vs K
- B vs K

Analgesia:

- L vs K
- A vs B
- A vs K
- B vs K

Statistics

All data was handled electronically and calculations performed using either Microsoft Excel 2000 or Graphpad Prism 3.0. Comparisons between continuous variables were made using either ANOVA with Bonferroni post test corrections or t-tests, depending which test was appropriate. Categorical data was analysed using the chi-squared test and correlation between independent variables was tested with Pearsons correlation coefficient. For all comparisons $p<0.05$ was considered significant.

Classification*	Physical Status
1	Normal, healthy
2	Mild systemic disease
3	Severe systemic disease that limits activity but is not incapacitating
4	Incapacitating systemic disease that is a constant threat to life
5	Moribund; not expected to survive 24 hours with or without an operation

* In the event of an emergency operation, precede the class number with an E.

Table 12. ASA Classification.(Kohn RLJ 1985)

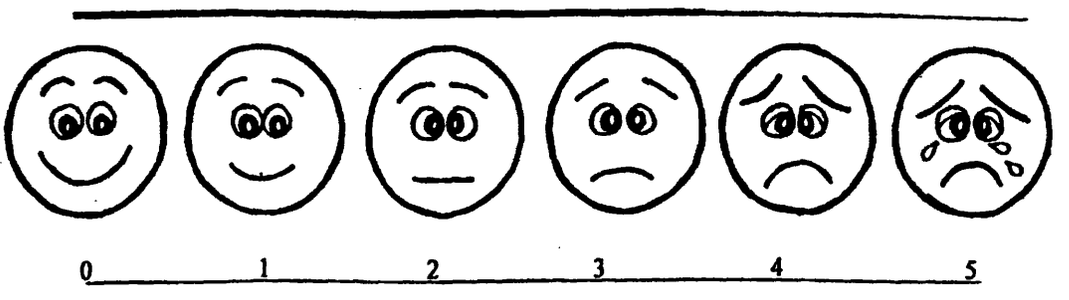

Figure 21. The Faces pain scale.(Wong D et al 1986)

Item	Behavior	Score	Definition
1. Cry	no cry	1	Child is not crying
	moaning	2	Child is moaning or quietly vocalising, silent cry
	crying	2	Child is crying, but the cry is gentle or whimpering
	scream	3	Child is in a full lunged cry; sobbing: maybe scored with or without complaint
2. Facial	composed	1	Neutral face expression
	grimace	2	Score only if definite negative facial expression
	smiling	0	Score only if definite positive facial expression
3. Verbal	none	1	Child is not talking
	other complaints	1	Child complains but not about pain
	pain complaints	2	Child complains about pain
	both complaints	2	Child complains about pain and other things
	positive	0	Child makes positive statements or talks about other things without complaint
4. Torso	neutral	1	Body (not limbs) is at rest: torso is inactive
	shifting	2	Body is in motion in a shifting or serpentine fashion
	tense	2	Body is arched or rigid
	shivering	2	Body is shuddering or shaking involuntarily
	upright	2	Child is in vertical or upright position
	restrained	2	Body is restrained
5. Touch	not touching	1	Child is not touching or grabbing at the wound
	reach	2	Child is reaching for but not touching the wound
	touch	2	Child is gently touching wound or wound area
	grab	2	Child is grabbing vigorously at wound
	restrained	2	Child`s arms are restrained
6. Legs	neutral	1	Legs maybe in any position but relaxed
	squirming/kicking	2	Definite uneasy or restless movements in the legs and/or striking out with the foot or feet
	drawn up/tensed	2	Legs tensed and/or pulled up tightly to body
	standing	2	Standing, crouching or kneeling
	restrained	2	Child`s legs are being held down

Table 13. CHEOPS Score - give one score for each of the categories and add together to give a total score.(McGrath PJ et al 1985)

	Description
1	Awake
2	Drowsy
3	Asleep but moves spontaneously
4	Asleep responds to stimulation
5	Hard to rouse

Table 14. Sedation Score.

4.3 Clinical study - Results

Ninety eight patients were recruited into the study. Data was not recorded from two of these patients. One patient was given an incorrect dose of the opioid to be studied whilst the other initially consented to the trial but then withdrew their consent on re-attendance for surgery after the procedure was postponed on their first attendance. Thus data was collected from a total of 96 patients. Of these patients 48 received morphine and 48 received codeine.

4.3.1 Demographic data

The demographic data for the patients are shown in table 15. There was no random difference between the two groups for sex, age and surgical time, taking a significance level of $p<0.05$. However, there was a significant difference between the two groups with respect to weight with $p = 0.044$.

Table 16 shows a breakdown of the ethnic groups of the patients randomised to the two arms of the study. The wide diversity in each of the groups reflects the multicultural society that provides the patient base for an inner-city London hospital.

4.3.2 Time to first analgesia

The decision to administer supplementary analgesia to a patient was made on clinical grounds by the anaesthetist or nurse responsible for the care of the child. The time to this analgesia was recorded in minutes from the time of injection of the opioid being studied. Comparisons between the two groups were made using Kaplan-Meyer survival curves as shown in figure 22. Two comparisons were made, the first using 4 hours (240 minutes) as the time at which the patient had "survived" if no rescue analgesia had been given, and the second using 2 hours (120 minutes).

In the codeine group 20 of the 48 (41.7%) patients required extra analgesia within 4 hours from the time of injection compared with 9 of the 48 (18.8%) patients in the

morphine group. Looking at a time period of 2 hours after injection the number of patients who required extra analgesia was decreased, 8 (16.7%) in the codeine group and 2 (4.2%) in the morphine group.

There was a significant difference between the two groups in both comparisons. Using a 4 hour survival time $p = 0.0094$ and using a 2 hour survival time $p = 0.045$.

4.3.3 Pain Scores

Both the CHEOPS observational pain score and the Faces self-report pain score were treated as continuous data. For comparison means at each time point were taken and analysis was performed using unpaired t-tests, with $p<0.05$ considered significant.

Behavioural scores at rest

In the recovery room, before any child had been given any further analgesia, there was no significant difference between the two groups for CHEOPS pain score at rest. There was also no significant difference between the two groups for pain scores recorded at the other time points.

However, in both groups a proportion of the patients required analgesia before 4 hours postoperatively. If supplementary analgesia was given to a child, they were excluded from further assessment. Thus figure 23 shows a comparison of the CHEOPS pain scores, at rest, between the groups with the children subdivided into further subgroups depending on whether further analgesia was given and if so, when it was given.

Analysis shows there was a significant difference between the pain scores of the children given analgesia in recovery in the codeine group compared with the other children in the codeine group and the corresponding children in the morphine group ($p<0.05$ in all cases). Also pain scores were higher in recovery than at other time points in both the codeine and morphine patients in the children not requiring extra analgesia

at any time point ($p<0.05$). In all other comparisons between the different subgroups there was no significant differences either within a group or between the two groups.

Faces scores at rest

To allow for recording of the Faces pain score the child must be willing to cooperate, although the children were compliant at the majority of time points on occasions an individual child would not cooperate at an individual time point. In this case no score was recorded at that particular point. If a child was asleep, they were not awoken, and a score of 1 was recorded.

In the recovery room, before any child has been given any further analgesia, there was no significant difference between the two groups for the Faces pain score at rest. There was also no significant difference between the two groups for pain scores recorded at the other time points.

As for the CHEOPS score, the children have been subdivided into further subgroups depending on whether further analgesia was given and if so, when it was given. This is shown graphically in figure 24.

Analysis between these groups shows there was a significant difference between the pain scores of the children given analgesia in recovery in the codeine group compared with the other children in the codeine group ($p<0.05$ in all cases) though there is no difference with the corresponding children in the morphine group. Pain scores are also higher in recovery than at other time points in the morphine patients in the children not requiring extra analgesia at any time point ($p<0.05$). In all other comparisons between the different subgroups there was no significant differences either within a group or between the two groups.

Pain scores on swallowing

To allow for the pain scores to be repeated with the child actively swallowing, at each of the times where pain scores were recorded at rest, the child must be awake and compliant. As this was not always the case, not every child could be scored at every time point. Also, as previously, once a child had been given extra analgesia no further data was collected.

Thus analysis could only be carried out on a smaller set of data compared to pain scoring at rest. When comparing the pain scores to those at rest only the scores from patients able to give both scores were used i.e. paired data. Analysis was carried out using paired and unpaired t-tests, with $p<0.05$ considered significant.

CHEOPS scores

In the recovery room, before any child has been given any further analgesia, there is no significant difference between the two groups for CHEOPS pain score on swallowing. There is also no significant difference between the two groups for pain scores recorded at the other time points.

However, in both the codeine group, at all time points, and in the morphine group, at all time points apart from 2 hours, the pain scores are higher on swallowing than at rest, with larger differences seen between the two pain scores in the codeine group compared with the morphine group.

Faces scores

In the recovery room, before any child has been given any further analgesia, there is no significant difference between the two groups for Faces pain score on swallowing. There is also no significant difference between the two groups for pain scores recorded at the other time points.

However, in both the codeine group, at all time points, and in the morphine group, at all time points apart from 4 hours, the pain scores are higher on swallowing than at rest, with larger differences seen between the two pain scores in the codeine group compared with the morphine group.

Comparison of pain scores

Figure 25 shows the correlation of the CHEOPS and Faces pain scores taken at rest and on swallowing in recovery. Patients from both groups were combined for this comparison. These data were analysed using Pearsons correlation. There is an association between the two scores at rest with the correlation coefficient (r) = 0.5, 95% confidence intervals (CI) = 0.32 - 0.64 and $p<0.0001$. There is a smaller association between the scores on swallowing with r = 0.42, CI (95%) = 0.21 - 0.59 and $p=0.0002$.

4.3.4 Side-effects

Postoperative vomiting

Figure 26 shows a comparison between the morphine and codeine groups for the number of patients who vomited in each of the time epochs studied. These data were studied using chi-squared tests by comparing the proportions of patients who vomited with those who did not in each of the drug groups at each of the time epochs, with $p<0.05$ considered significant.

There was no significant difference between the two groups in the proportion of patients who vomited at <1, 1-2 and 4-8hrs but there was a significant difference at 2-4hrs with $p<0.002$.

Looking at the total number of patients who vomited in the first four hours postoperatively, 56.3% of patients in the morphine group vomited compared with 29.2% of patients in the codeine group. Analysing these data, again using a chi-squared

test to compare the proportions, shows a significant difference between the two groups with $0.01<p<0.02$.

Anti-emetics were only administered after a child had vomited and then only if the nurse or anaesthetist caring for the child felt their use was justified. Only 3 patients in each group were given anti-emetics in the first four hours postoperatively. Removing these patients from the analysis in subsequent time epochs does not alter the results.

Sedation scores

Figure 27 shows comparisons between the two groups for sedation scores taken at each of the time points for data collection. Analysis of the data, using an unpaired t-test at each time point, shows there is no significant difference in sedation score between the two groups in recovery or at 1, 3 and 4 hours. However, there is a significant difference between the two groups at 2 hours with $p = 0.005$

Removing the 4 patients in the codeine group who were given morphine prior to recording the data at 1 hour postoperatively and re-analysing the data does not alter the results at any time point.

Respiratory rates

Figure 28 shows comparisons between the two groups for respiratory rate taken at each of the time points for data collection. Analysis of this data, using an unpaired t-test at each time point, shows there is no significant difference in respiratory rate between the two groups at any of the time points.

Time to oral intake

The time to oral in take was measured from the end of surgery to the time of first taking oral fluids. A comparison between the two groups using an unpaired t-test showed there was no significant difference between the two groups.

Further complications

Perioperative

Five patients in the codeine group and none in the morphine required replacement of the laryngeal mask airway with an endotracheal tube. On three occasions this was due to being unable to maintain the airway on insertion of the surgical gag, one was due to being unable to provide a satisfactory airway in the anaesthetic room and on the other occasion the tube was placed after an episode of laryngospasm.

One patient in each group had a prolonged stay in recovery (>1 hour) due to taking a long time to awaken after the anaesthetic.

Postoperative

One patient in each group had a postoperative haemorrhage that required a return to theatre some hours later. Two patient in the morphine group and one in the codeine group required intravenous fluids due to vomiting leading to poor oral intake and one morphine patient had significant swelling and pain the next day which delayed discharge for an extra day, which in all other cases was the morning after the procedure.

4.3.5 Plasma morphine and morphine metabolite levels

Figure 29 uses a scatter plot to show the comparisons between the codeine and morphine groups for the plasma levels of morphine and its glucuronide metabolites one

hour following the intra-muscular injection of the opioid. One sample from the codeine group was lost by the laboratory, thus in the codeine group there were 47 patients and in the morphine group there were 48 patients.

Measurement of plasma morphine and metabolite levels was performed using HPLC by Innes Kastner and Jonathan De-Lima at the Institute of Chilh Health, London. Analysis was done in two batches, 73 samples in the first batch and 22 in the second. The limits of detection were 5ng/ml for morphine and 10ng/ml for M-6-G in both batches. However, for M-3-G, the limit of detection was 30ng/ml in the first batch and 60ng/ml in the second.

When analysing the data it was necessary to assign a value to all the results that were not detected (ND) to allow statistical comparison, as they were numerous and could not all be considered to be zero. Assuming a normal distribution for the results, the area to the left of the limit of detection (the end of the tail) approximates to a right angled triangle and all the ND samples will fall within this triangle. To take a value for ND that would split the area of the triangle in half, requires taking a point that is two-thirds of the way between 0 and the limit of detection i.e. on average half of these ND samples would be above this value and half would be below. Thus for morphine the ND value was taken as 3.3ng/ml, for M-6-G it was 6.7ng/ml and for M-3-G it was 20ng/ml for the first batch and 40ng/ml for the second.

These data were then analysed using unpaired t-tests with $p<0.05$ being considered significant. There was a significant difference between the morphine and codeine groups in all comparisons with $p<0.0001$ for morphine and M-3-G and $p=0.0021$ for M-6-G.

Comparison of morphine levels with pain scores

The pain scores in recovery were recorded at the same time as blood was taken for analysis of plasma morphine and morphine metabolite levels. For each of the drug groups the plasma morphine levels were correlated with both the observational and the self-report pain scores, at rest, using Pearsons correlation (figure 30). None of the

comparisons showed a significant association between plasma morphine levels and pain score:

Comparison	r	CI (95%)	p
Group M vs CHEOPS	-0.29	-0.53 - -0.002	=0.05
Group C vs CHEOPS	0.12	-0.18 - 0.39	>0.05
Group M vs Faces	-0.13	-0.41 - 0.18	>0.05
Group C vs Faces	0.01	-0.29 - 0.31	>0.05

There was also no correlation between plasma morphine levels and both the observational and the self-report pain scores on swallowing (figure 31):

Comparison	r	CI (95%)	p
Group M vs CHEOPS	-0.11	-0.41 - 0.21	>0.05
Group C vs CHEOPS	0.16	-0.15 - 0.44	>0.05
Group M vs Faces	-0.09	-0.41 - 0.25	>0.05
Group C vs Faces	0.06	-0.26 - 0.37	>0.05

Similarly no correlation between was found between morphine levels at one hour and time to 1st analgesia in either group. For the morphine group $r = 0.11$ (CI = -0.19 – 0.39 and $p = 0.45$) and for the codeine group $r = 0.09$ (CI = 0.21 – 0.37 and $p = 0.55$).

4.3.6 Patient genotypes

Tables 17 and 18 show the genotypes and possible phenotypes for the patients in the morphine and codeine groups respectively. The phenotype of each patient was postulated from the known activity of each gene present. If a patient was homozygous for polymorphisms known to show no activity they were classified as a poor metaboliser, whilst a combination of a polymorphism of reduced activity with one of no activity classified the patient as an intermediate/poor metaboliser. An intermediate metaboliser was a patient with either a combination of two polymorphisms with reduced activity or a combination of one normal polymorphism with one of no activity and an extensive metaboliser was a patient who was either homozygous for the normal gene or who had a combination of a normal polymorphism with one of reduced activity.

The number of each phenotype in the respective groups were:

Phenotype	Group M	Group C
Poor	2	2
Intermediate/poor	2	5
Intermediate	5	10
Extensive	39	29

The codeine group showed more variability in the types of phenotype present. However, there was no link between the phenotype and the time to 1st analgesia. Of the two PMs in the codeine group one patient required further analgesia at 187 minutes and the other required no further analgesia in the first four postoperative hours. Similarly when looking at the intermediate/poor metabolisers in the codeine group one patient required further analgesia at 60 minutes but the other four required no further analgesia in the first four postoperative hours.

In the codeine group, of the 8 patients who required analgesia in the first two postoperative hours, 5 were EMs, 2 were IMs and 1 was an intermediate/poor metaboliser. Similarly, of the 20 patients who required analgesia in the first four postoperative hours, 13 were EMs, 4 were IMs, 1 was an intermediate/poor metaboliser and 1 was a PM, with no sample being provided from the other patient.

In the morphine group there was also no link between the phenotype and the time to 1st analgesia. Of the Of the two PMs, one patient required further analgesia at 237 minutes and the other required no further analgesia in the first four postoperative hours. Both the intermediate/poor metabolisers required no further analgesia in the first four postoperative hours. The two patients who required analgesia in the first two postoperative hours were both EMs as were eight of the nine patients who required analgesia in the first four postoperative hours, the other being a PM.

Phenotype and plasma morphine levels

In the codeine group, morphine and/or its glucuronide metabolites were not detected in the plasma of either of the two PMs at one hour after injection. Of the 5 intermediate/poor metabolisers, in two morphine and/or its metabolites were not

detected (including the one patient to require supplementary analgesia) whilst two showed only low levels of M-3-G and the other one only low levels of M-6-G.

Following codeine, morphine and/or its metabolites were not detected in 17 patients. Of these 8 were EMs, 4 were IMs, 2 were intermediate/poor metabolisers and 2 were PMs, with no sample being provided from the other patient. Thus of the other 31 patient in whom morphine and/or its metabolites were detected 21 were EMs, 6 were IMs and 3 were intermediate/poor metabolisers.

In the morphine group, morphine and its metabolites were recorded from all the patients regardless of their phenotype.

	Group C - codeine (n = 48)	Group M - morphine (n = 48)
Sex* (male/female)	24/24	23/25
Age* (years)	7.31 (+/- 2.98)	6.23 (+/- 2.58)
Weight (kg)	32.39 (+/- 19.2)	25.79 (+/- 11.49)
Surgical time* (mins)	33.52 (+/-10.7)	33.06 (+/- 11.48)

Table 15. Demographic data for the 96 patients recruited into the trial. * = no significant difference between the two groups taking $p<0.05$ as significant. There is a significant difference between the two groups in terms of weight ($p = 0.044$).

Ethnic Group	Group M (n = 48)	Group C (n = 48)
Caucasian	21	15
Asian	12	10
Afro- Caribbean	5	11
Turkish	5	3
Arab	0	2
Caucasian/Asian	3	1
Caucasian/Afro-Caribbean	1	4
Caucasian/Arab	1	1
Asian/Afro-Caribbean	0	1

Table 16. Comparison between the composition of the ethnic groups randomised to receive codeine and morphine.

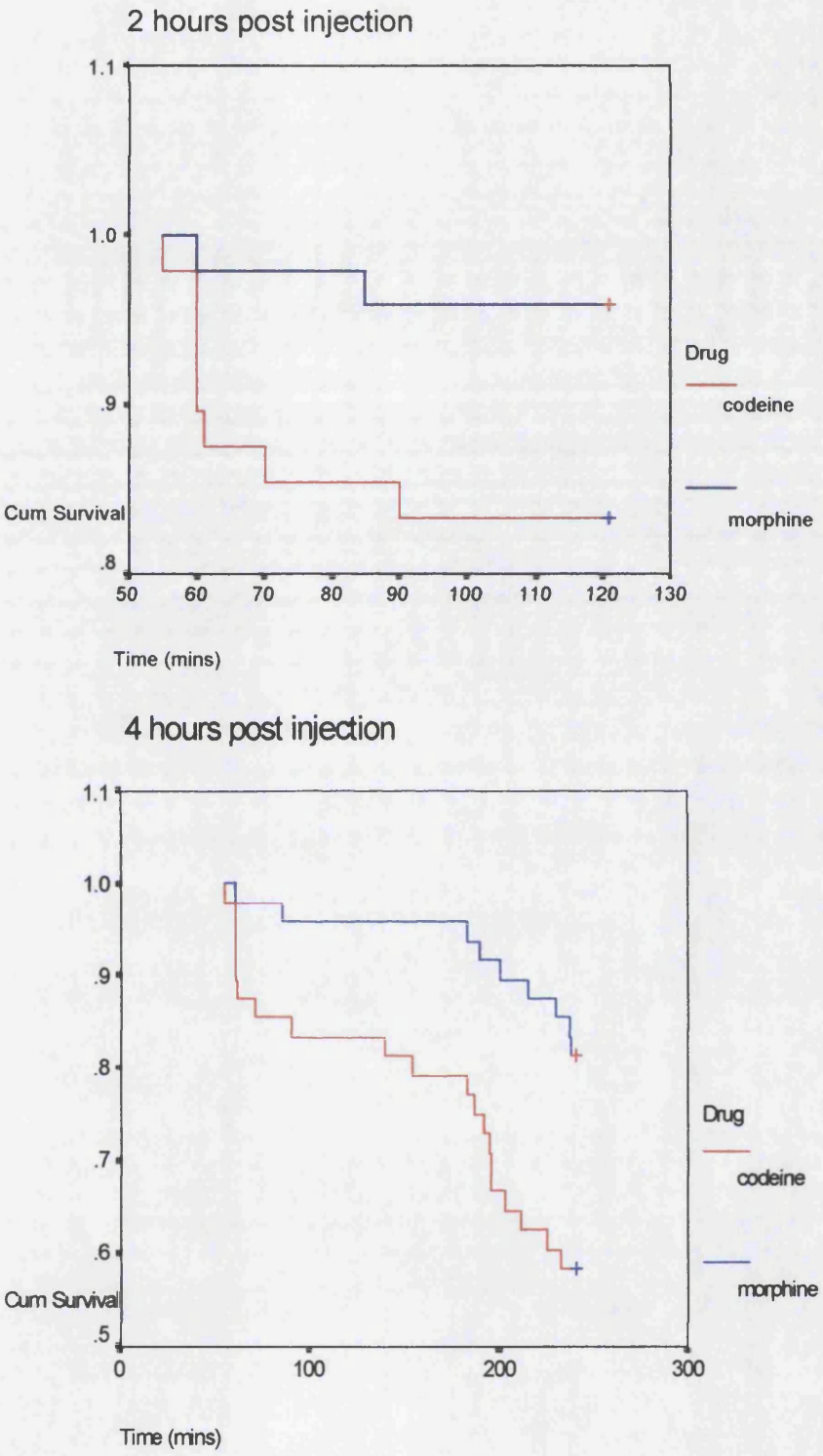

Figure 22. Comparison between the codeine and morphine patients requiring extra analgesia using Kaplan-Meyer survival curves, with survival times taken as 2 and 4 hours post injection.

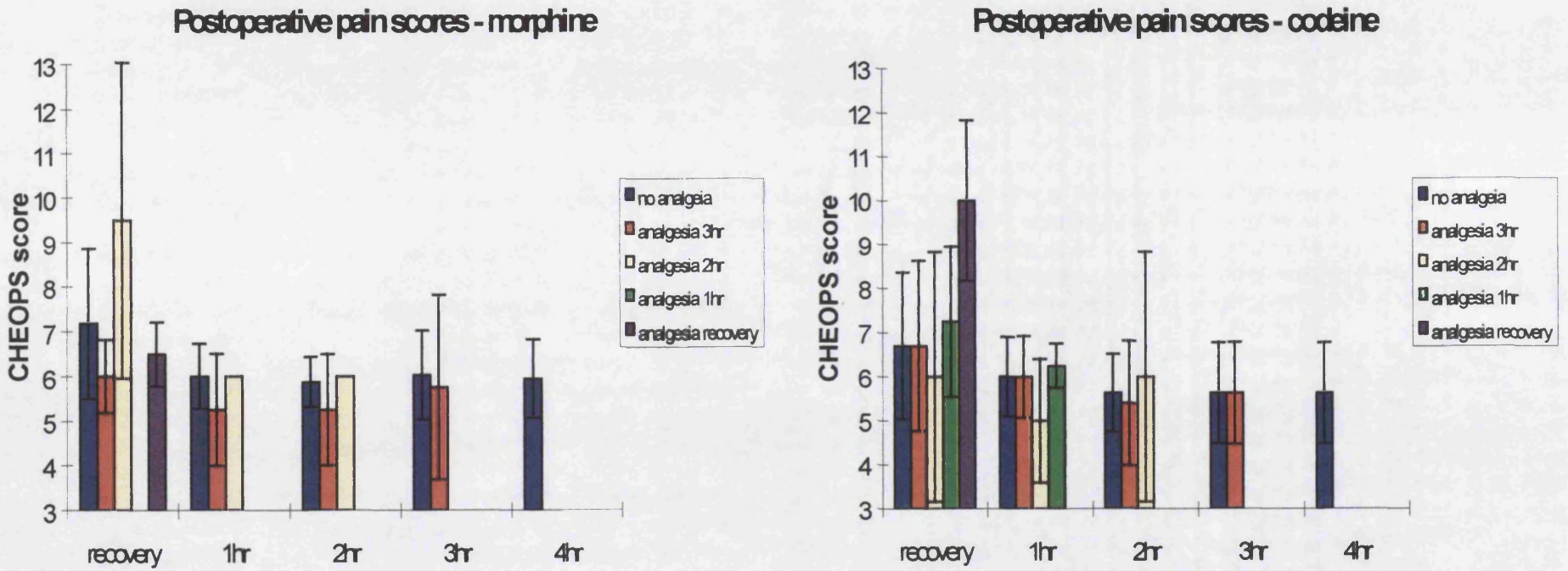

Figure 23. Comparison of postoperative pain scores at rest, using the CHEOPS pain scale, between the morphine and codeine groups.

Displayed are means and standard deviations. T-tests were used to analyse these data (see text).

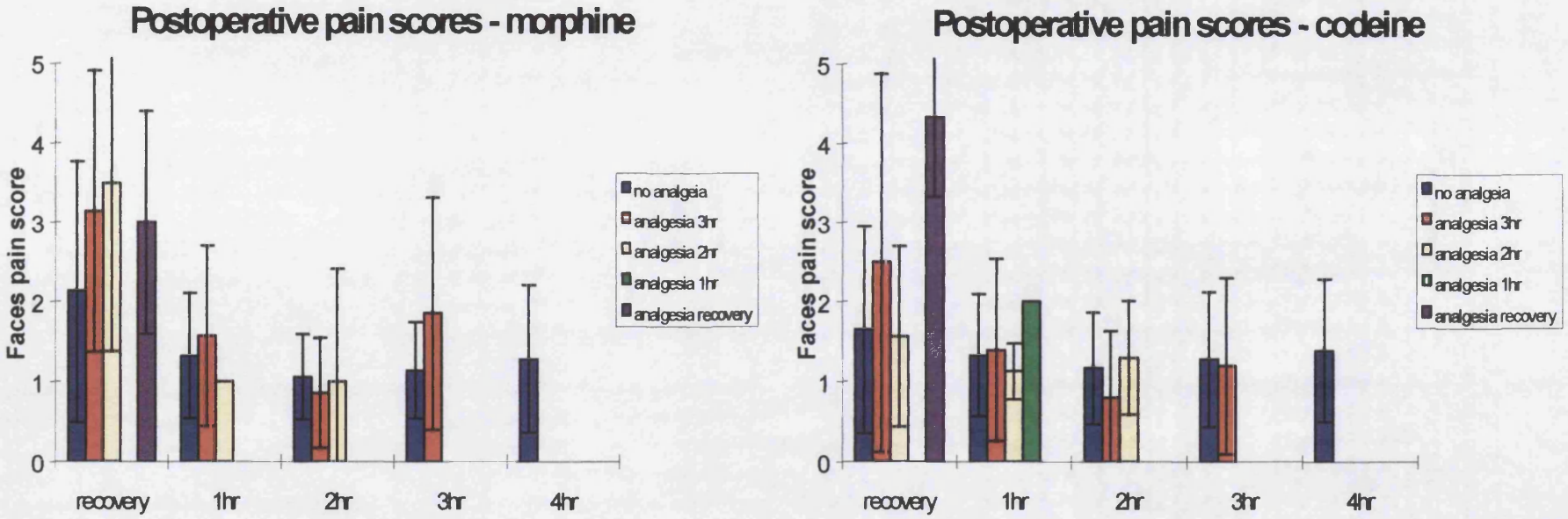

Figure 24. Comparison of postoperative pain scores at rest, using the Faces pain scale, between the morphine and codeine groups. Displayed are means and standard deviations. T-tests were used to analyse these data (see text).

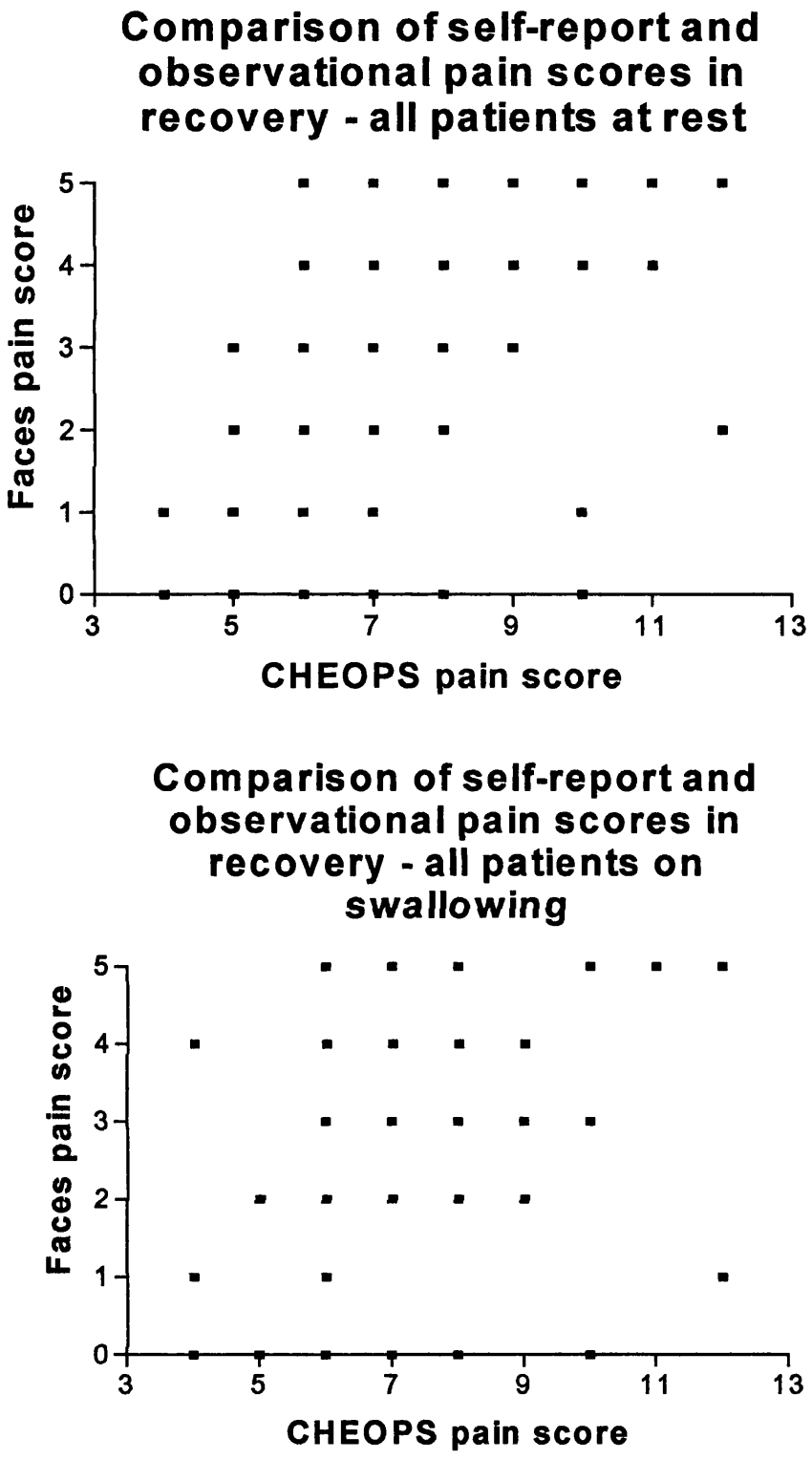

Figure 25. Correlation of observational and self-report pain scoring for all patients at rest and on swallowing from scores taken in the recovery room.

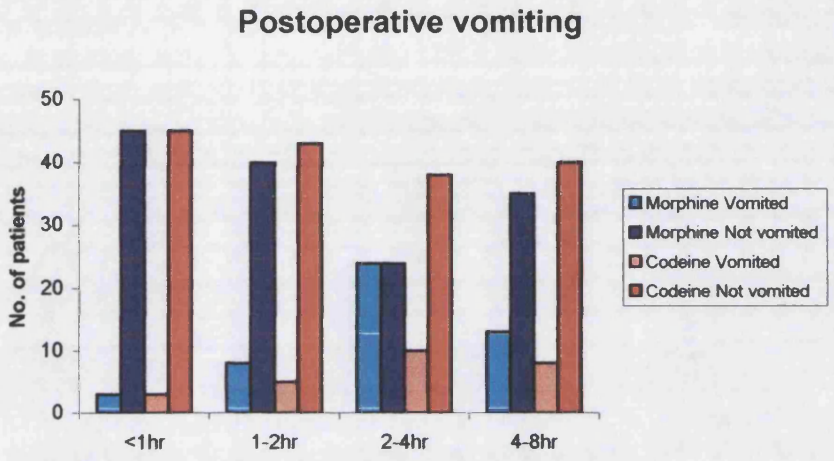

Figure 26. Comparison of the number of patients who vomited between the codeine and morphine groups in each postoperative time epoch.

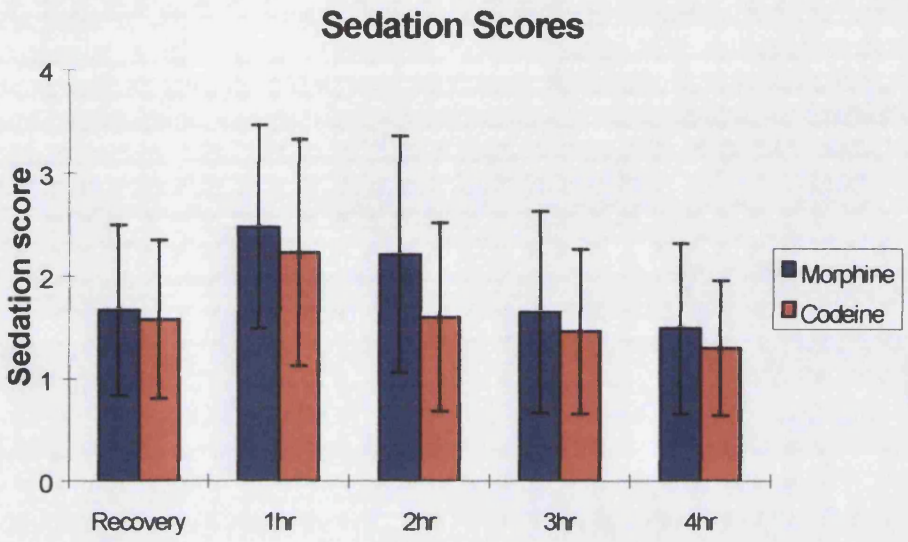

Figure 27. Comparisons of postoperative sedation scores between the morphine and codeine groups. Means plus standard deviations shown. Analysis was performed between the two groups at each time point using a t test. There was no sinificant difference in the scores in recovery and at 1, 3 and 4 hours. There was a significant difference between the two groups at 2 hours with $p = 0.005$.

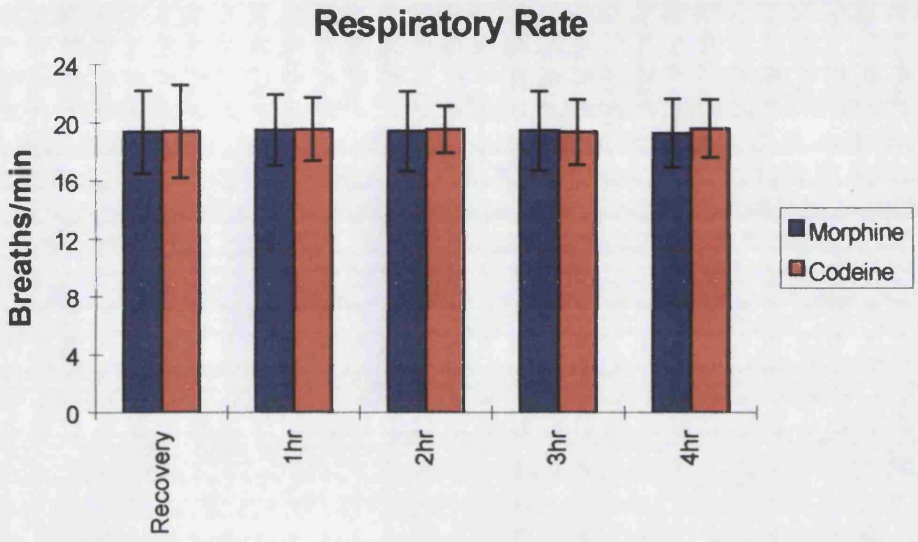

Figure 28. Comparisons of postoperative respiratory rates between the morphine and codeine groups. Mean plus standard deviation displayed. Analysis was performed using a t-test at each time point and there was no significant difference between the two groups at any time point.

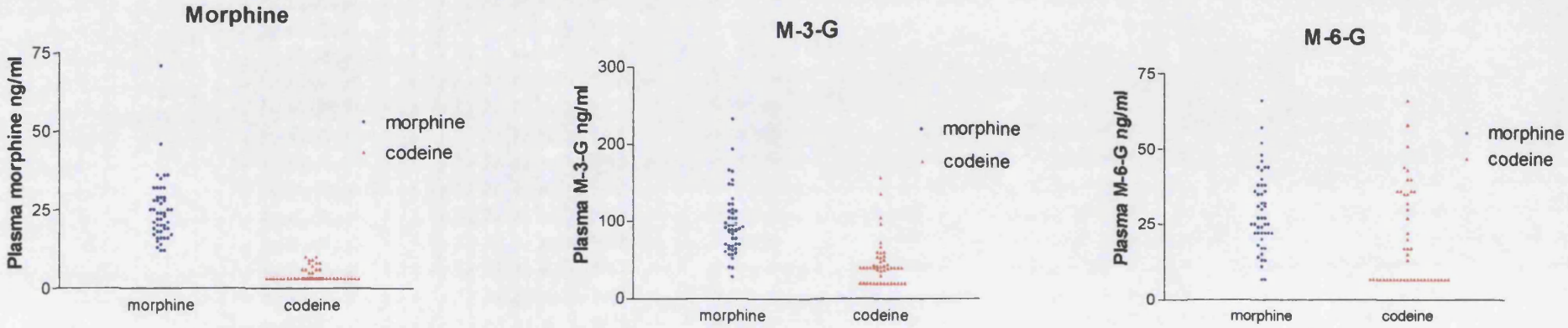

Figure 29. Scatter plots showing the comparisons of plasma levels of morphine and its glucuronide metabolites between the codeine and morphine groups one hour following intra-muscular injection of the opioid.

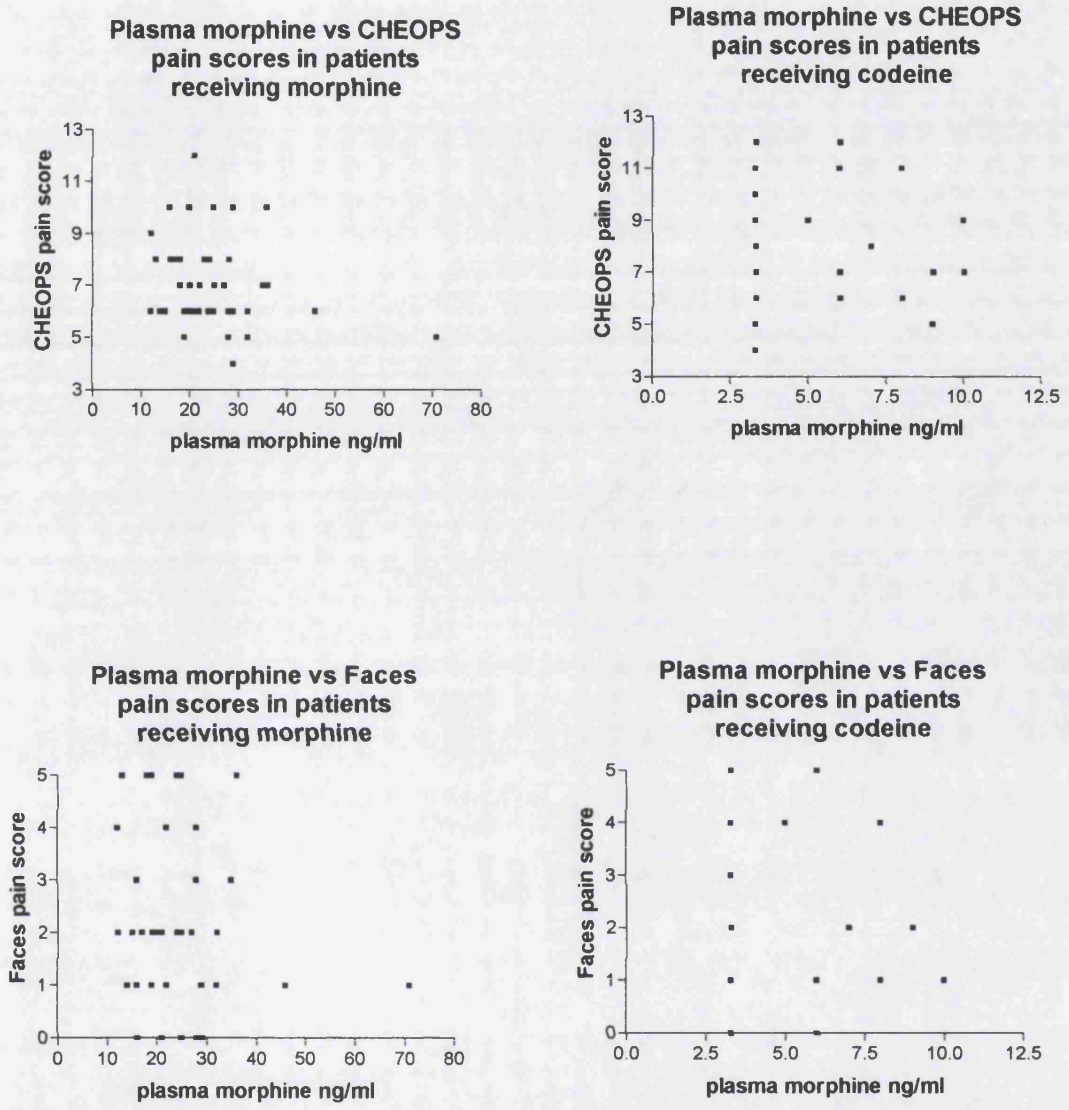

Figure 30. Correlations of plasma morphine levels with pain scores in recovery, at rest, in both the codeine and morphine groups.

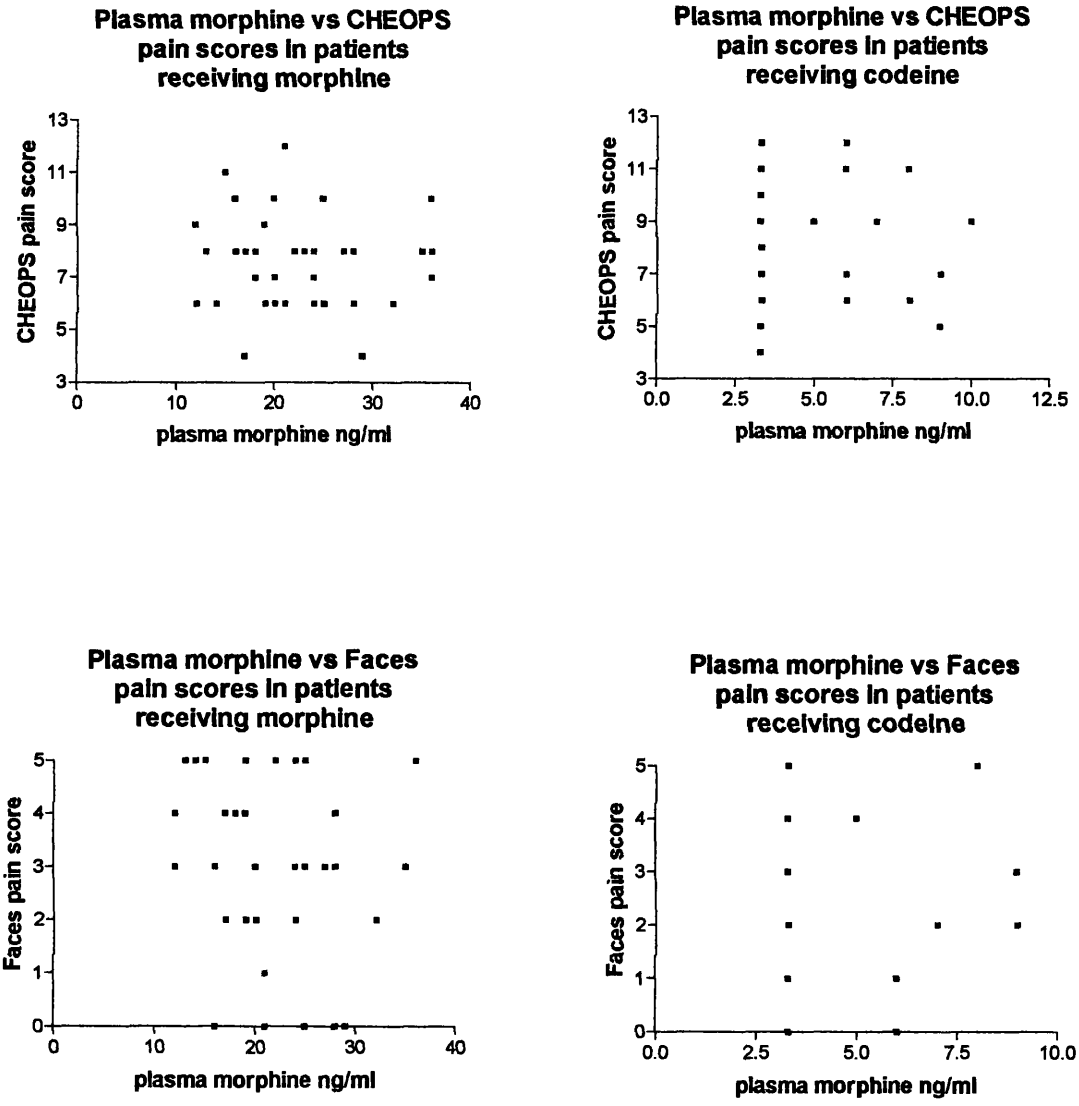

Figure 31. Correlations of plasma morphine levels with pain scores in recovery, on swallowing, in both the codeine and morphine groups.

Patient No.	Genotype	Possible Phenotype	Time to 1st analgesia (mins)
1	*1/*4	Intermediate	>240
2	*1/*1	Extensive	>240
3	*1/*17	Extensive	>240
4	*1/*9	Intermediate	>240
5	*1/*1	Extensive	200
6	*1/*1	Extensive	85
7	*2/*2	Extensive	>240
8	*2/*2	Extensive	>240
9	*2/*10	Intermediate	>240
10	*1/*2	Extensive	>240
11	*1/*1	Extensive	215
12	*2/*2	Extensive	>240
13	*2/*2	Extensive	>240
14	*2/*2	Extensive	190
15	*2/*2	Extensive	183
16	*2/*2	Extensive	>240
17	*4/*4	Poor	>240
18	*1/*2	Extensive	>240
19	*1/*4	Intermediate	>240
20	*1/*2	Extensive	>240
21	*1/*1	Extensive	>240
22	*2/*2	Extensive	>240
23	*1/*17	Extensive	>240
24	*1/*1	Extensive	>240
25	*1/*10	Extensive	>240
26	*1/*2	Extensive	230
27	*2/*4	Intermediate/Poor	>240
28	*4/*4	Poor	237
29	*1/*2	Extensive	>240
30	*2/*2	Extensive	>240
31	*2/*3	Intermediate/Poor	>240
32	*1/*2	Extensive	60
33	*1/*2	Extensive	>240
34	*1/*1	Extensive	>240
35	*2/*2	Extensive	>240
36	*1/*1	Extensive	>240
37	*2/*2	Extensive	>240
38	*1/*2	Extensive	>240
39	*1/*4	Intermediate	>240
40	*2/*2	Extensive	>240
41	*1/*2	Extensive	>240
42	*1/*1	Extensive	238
43	*1/*2	Extensive	>240
44	*1/*1	Extensive	>240
45	*1/*1	Extensive	>240
46	*1/*2	Extensive	>240
47	*1/*2	Extensive	>240
48	*1/*1	Extensive	>240

Table 17. Genotypes and Phenotypes of the patients receiving morphine.

Patient No.	Genotype	Possible Phenotype	Time to 1st analgesia (mins)
1	*1/*17	Extensive	>240
2	No Sample		195
3	*1/*2	Extensive	>240
4	*1/*2	Extensive	>240
5	*4/*4	Poor	>240
6	*1/*1	Extensive	>240
7	*1/*4	Intermediate	>240
8	*2/*2	Extensive	>240
9	*2/*4	Intermediate/Poor	>240
10	*9/*10	Intermediate/Poor	60
11	*2/*17	Intermediate	70
12	*1/*1	Extensive	60
13	*1/*4	Intermediate	>240
14	*2/*4	Intermediate/Poor	>240
15	*1/*1	Extensive	>240
16	*1/*1	Extensive	140
17	*1/*4	Intermediate	>240
18	*1/*4	Intermediate	>240
19	*1/*1	Extensive	225
20	*1/*10	Extensive	60
21	*1/*1	Extensive	155
22	*2/*2	Extensive	>240
23	*1/*2	Extensive	>240
24	*1/*3	Intermediate	>240
25	*1/*17	Extensive	>240
26	*1/*4	Intermediate	203
27	*1/*2	Extensive	>240
28	*1/*4	Intermediate	55
29	*1/*10	Extensive	90
30	*17/*17	Intermediate	>240
31	*1/*1	Extensive	>240
32	*2/*4	Intermediate/Poor	>240
33	*1/*10	Extensive	>240
34	*17/*17	Intermediate	>240
35	*1/*2	Extensive	183
36	*1/*1	Extensive	60
37	*2/*2	Extensive	195
38	*1/*9	Intermediate	232
39	*2/*2	Extensive	194
40	*1/*1	Extensive	>240
41	*2/*2	Extensive	>240
42	*1/*1	Extensive	>240
43	*1/*2	Extensive	>240
44	*1/*1	Extensive	192
45	*4/*10	Intermediate/Poor	>240
46	*4/*4	Poor	187
47	*2/*2	Extensive	211
48	*2/*2	Extensive	61

Table 18. Genotypes and Phenotypes of the patients receiving codeine.

4.4 Clinical study – discussion

The results from this study show that overall morphine and codeine appear to provide equivalent analgesia at the doses prescribed with a decreased incidence of postoperative vomiting in the patients receiving codeine. These results are in agreement with previous work looking at the effects of codeine and morphine, at the same doses, also in children undergoing adenotonsillectomy.(Semple D et al 1999) However, on an individual patient basis, in comparison with morphine, codeine shows more variability in its effect and there is a small group of patients in whom it appears to provide little analgesia.

4.4.1 Analgesic Efficacy

Time to first analgesia

More patients required rescue analgesia following codeine than morphine in the first four hours postoperatively (41.7% vs 18.8%). This suggests that either codeine is not providing adequate analgesia in a large proportion of patients and that the effect is very variable between patients or that the duration of action of codeine is shorter that than morphine when given intra-muscularly. The half lives of both drugs following intra-muscular injection are 2-3 hours and the usual dosing regimen for both drugs for intra-muscular injection is 4-6 hourly which would suggest that the duration of action of each drug is similar and would potentially be effective for 4 hours after administration.(Dolhery C 1999; British National Formulary 1999; Kart T et al 1997a)

In an attempt to exclude duration of action as a variable, comparisons were also made 2 hours postoperatively. These again show a difference ($p=0.045$) between the two groups with more patients in the codeine group requiring rescue analgesia in the first 2 hours. Thus there appears to be a subgroup of patients who receive little or no analgesic benefit from codeine in comparison with morphine. This variability in efficacy between patients may be a possible explanation for the high NNT of codeine in comparison with other analgesic agents and preparations where codeine is used in combination with other drugs. The variability in effect of codeine seen is also not inconsistent with the hypothesis that differences in CYP2D6 activity between patients may lead to variable

metabolism to morphine and thus variable efficacy.

Pain Scores

The variability in effect of codeine compared with morphine was not demonstrated by either the self-report or the observational pain scoring. Both scores showed there was no difference in analgesic efficacy between the two groups. Rescue analgesia was given as a result of clinical assessment and the need for supplementary analgesia did not correlate with the pain scores

When analyzing a group of patients as a whole, in which only a small proportion exhibit no or little benefit, it is not surprising that the pain scores are similar. For the majority of patient the scores were similar in both groups and thus the occasional patient with a higher score would only marginally affect the mean score. This may also be a reason why previous studies looking at the efficacy of codeine, using small numbers of patients and not looking for individual patient variability, have not questioned its adequacy in some patients.

Pain is a subjective experience making accurate assessment and comparisons between patients very difficult. These problems are compounded in children due to age related differences in the parameters used to assess pain. Thus no single pain assessment tool has been shown to be entirely accurate at all ages and in all situations. In an attempt to overcome these problems a self-report and a behavioural pain score were used. Limitations of both tools were highlighted during this trial.

Children are said to have cognitive development by 3 -4 years sufficient to allow self reporting of pain and the use of self-report pain tools has been validated at these ages.(Harbeck C et al 1992) Though in younger children it has been recognised that they often select at the extremes of pain scales, ignoring the midsections. (Franck LS et al 2000) Childrens behavioural responses to pain also change with age and there was no score suitable across the age range studied. It is also difficult to discriminate between behaviours corresponding to pain and those relating to generalised distress and agitation.(Franck LS et al 2000) Objectively, during the study, it was felt by the

assessors that some children tended to pick their favourite faces and that clinical assessment of pain did not always correlate with the pain score assessment.

The problems associated with both these scores may also be a reason why the variability in effect of codeine was not highlighted by the pain scoring, and may also be a reason why this has not been shown in previous work using codeine in children. The limitations of these pain scores is also highlighted by the weak associations seen when correlating the two pain scores taken in recovery at rest and on swallowing ($r=0.5$ and $r=0.42$ respectively). These correlations also highlight differences in patient and health care worker assessments of pain seen in previous studies.(Romsing J et al 1996; LaMontagne LL et al 1991)

Pain on swallowing

Swallowing was used as a model for movement at the site of surgery. Using both pain tools, scores were higher on swallowing than at rest with larger differences between the two scores seen in the patients receiving codeine compared with those receiving morphine. This confirms previous studies showing that pain at rest is more sensitive to opioids that pain on movement.(Cooper BY et al 1986; Eriksson MBE et al 1982; Beecher HK 1957) It also may suggest that codeine is less effective than morphine for pain associated with movement in the presence of tissue injury.

Use of NSAIDs

Whichever method of assessing pain is used the results are complicated by the concurrent use of NSAIDs. It is currently accepted clinical practice to adopt a balanced, or multi-modal, approach to analgesia, using techniques that act on different areas of the pain pathways, as there is no single method that is efficacious in all situations and use of more than one drug allows doses to be decreased and thus reduce unwanted side-effects.(Kehlet H et al 1999)

Commonly employed perioperative analgesic techniques for adenotonsillectomy in

children involve the use of opioids with simple analgesics such as NSAIDs and paracetamol plus, in some cases, local anaesthetic infiltration. It was important to strike a balance between an acceptable analgesic technique and the introduction of too many confounding factors, a problem encountered in many clinical drug trials. It would have been unacceptable to design a study protocol in which an intramuscular injection of opioid was the only peri-operative analgesia. Thus, it was decided to add in one further analgesic drug, NSAIDs.

NSAIDs have been shown to have opioid sparing effects for postoperative pain following tonsillectomy in adults and children.(Salonen A et al 2001; Sutters KA et al 1995) In one study, comparison of ketorolac 0.9mg/kg with morphine 0.1mg/kg in children undergoing tonsillectomy showed that the analgesic effect from the two drugs was similar.(Watcha MF et al 1992) Whilst they do not totally remove the need for opioid analgesia in all patients, the amount of opioid required will vary between patients and may not be needed at all in some patients. In children with severe obstructive sleep apnoea the use of opioids is contraindicated due to their sedatory effects and analgesic regimens usually involve the use of simple analgesic agents and local infiltration with good analgesic effect seen in many of the patients. Thus the use of NSAIDs in this trial will have an effect on the efficacy of the two opioids, they will reduce the opioid requirements of the patients and may mask some of the variation in opioid effect between the children.

4.4.2 Opioid related side-effects

Vomiting

Vomiting is a common problem following adenotonsillectomy in children with an incidence of between 30 and 75%.(Gunter JB et al 1995; Kermode J et al 1995)The cause of this vomiting is multi-factorial and may include stimulation of laryngeal and pharyngeal reflexes, gastric irritataion due to swallowed blood, anaesthetic agents and opioid administration.(Ved SA et al 1996; Weinstein MS et al 1994) Vomiting is unpleasant which may cause distress to both the child and the parents, and may also delay fluid intake potentially leading to dehydration and tonsillar bed infection.

In the first four hours postoperatively 29.2% of the patients vomited in the codeine group compared with 56.3% of patients in the morphine group. This is in agreement with a previous study comparing morphine and codeine in children undergoing adenotonsillectomy which showed that 60 % of children in the morphine group and 30% of children in the codeine group vomited in the first six hours postoperatively.(Semple D et al 1999) There was no difference in vomiting between the two groups after 4 hours which suggests that it was likely to be an opioid realted effect as in the early postoperative period the only difference in anaesthetic and surgical management was the opioid used in each patient and the duration of action of both the opioids given intramuscularly is around 4 hours.(Dolhery C 1999; British National Formulary 1999)

Previous work in children undergoing tonsillectomy confirms the lower rates of vomiting associated with codeine compared with morphine. One study comparing codeine with ketorolac, an NSAID, showed similar vomiting rates for codeine (31%), as previously mentioned, that were comparable to the rates seen following ketorolac.(Splinter WM et al 1996) Comparison of morphine with ketorolac in children undergoing tonsillectomy demonstrated increased vomiting rates following morphine.(Gunter JB et al 1995; Watcha MF et al 1992) In a study where all the patients received morphine, the anti-emetic metoclopramide reduced vomiting rates in comparison with placebo by a similar magnitude to that seen with codeine.(Ferrarri LR et al 1992)

The doses of the opioids used in this study are considered to be equipotent from previous work.(Semple D et al 1999; Wallenstein SL et al 1961) Thus differences in vomiting rates would seem to be due to differences in the qualities of the opioids rather than dosage potencies. However, if plasma morphine and metabolite levels are considered, in the children following morphine the levels were significantly higher than in the children following codeine. Whether there is a relationship between plasma morphine levels and vomiting is unknown but this may be an explanation for the higher vomiting rates following morphine administration. Experimental studies in adult human volunteers have shown that opioid related side-effects were markedly increased in EMs compared with PMs following oral codeine at lower doses of 75-100mg, though when

higher doses of 170mg were used the rates were similar between the two groups suggesting a role for codeine itself, or another metabolite, in the production of side-effects.(Eckhardt K et al 1997; Poulsen L et al 1996) The results from this current study do not suggest a direct action of codeine itself as the rates of vomiting were significantly lower in the codeine group and this was associated with lower plasma morphine and metabolite levels.

After surgery the children were encouraged to drink as soon as possible. This may have affected the incidence of vomiting but this applied to both groups and the time to first oral intake of fluid was equivalent between the groups so it is unlikely to be a source of bias when comparing the two groups. No attempt was made to assess nausea in these children due to the difficulties in conveying the concept of nausea to children and the lack of an objective method to measure the symptom.

Sedation and respiratory depression

There was no difference between the two drug groups in terms of respiratory rate at any time point and sedation was only increased in the morphine group at 2 hours. This is in broad agreement with the previous study comparing codeine and morphine for tonsillectomy in which sedation scores did not vary between the two groups at any time.(Semple D et al 1999)

This suggests that using equipotent doses of codeine and morphine has similar effects on sedation and respiratory depression and thus refutes the argument that codeine is a safer drug to use in these children with regard to sedation and airway protection. Previous work has also shown that when comparing morphine with ketorolac no increased incidence of sedation is seen with the opioid.(Gunter JB et al 1995) One patient in each group had a prolonged time to emergence from anaesthesia, the reasons delayed emergence can be multi-factorial and are more likely to be due to the anaesthetic than any differences between the opiods.

Other complications

Of the five patients in the codeine group who required replacement of the laryngeal mask with an endotracheal tube, only in one patient, who experienced an episode of laryngospasm, could there be a possible role for the difference in opioid used, although laryngospasm has many possible etiologies. Of the four other patients, 3 had problems with the surgical gag and in the other the laryngeal mask did not provide a satisfactory airway in the anaesthetic room. These complications are very unlikely to be influenced by the opioid used.

One patient from each group had a significant postoperative haemorrhage that required a return to theatre. This is a rate of around 2% in each group which is in agreement with previous studies.(Gunter JB et al 1995; Handler SD et al 1986) This would also suggest that there was no increase in postoperative haemorrhage associated with the use of NSAIDs as has been shown in previous work.(Splinter WM et al 1996; Fitz-James I et al 1995) However, we did not record all incidences of bleeding, only those requiring surgery and thus the overall incidence of postoperative haemorrhage is not known and no real conclusions can be drawn.

4.4.3 Plasma morphine and morphine metabolite levels

Plasma levels of morphine and its glucuronide metabolites were significantly lower at one hour after injection in the codeine group than in the morphine group. This could be thought to be a possible explanation for the increased variability in the efficacy of codeine seen in comparison with morphine, however, the lower levels seen were consistent across all the patients in the codeine group and thus included both those requiring supplementary analgesia and those who did not.

There are potentially a number of possible explanations for these lower plasma levels and their relation to efficacy. Firstly the levels were recorded at only one time point, thus giving little or no idea of the levels at other times. Little is known about the pharmacokinetics of codeine in children following i.m. injection. A time of one hour was chosen on the basis of a study looking at i.m. versus rectal codeine for neurosurgery

in children which showed peak plasma levels of morphine between 30 and 120 minutes.(McEwan A et al 2000) The levels in that study were also low and in many cases morphine was not detected at all time points. Absorption of the drugs following the i.m. injection may also have affected the plasma levels seen at one hour, which may have been different for the two drugs, a problem that could have been compounded by the fact that the volumes used for injection were not equivalent between the drugs.

As previously discussed NSAIDs have an opioid sparing effect and their use will to some extent mask the differences between the two opioids in terms of efficacy. These results may also suggest that codeine itself or one of its other metabolites is also having an analgesic effect though as discussed in this thesis it seems likely that most if not all the analgesic efficacy of codeine is from its conversion to morphine.

Once again the results have shown that there is no correlation between plasma morphine levels and effect. When comparing plasma morphine levels with time to first analgesia and pain scores at rest and on swallowing no association was found. This is in agreement with previous work in humans.(Kart T et al 1997b; Millar AJW et al 1987; Lynn AM et al 1984; Nahata MC et al 1984) Thus, as has also already been discussed in this thesis, plasma levels may be unrelated to effect and other factors such as binding to opioid receptors and transduction, CSF morphine and metabolite concentrations, metabolism within the CNS and receptor density may all have an important effect on efficacy. It is also known that codeine crosses the BBB more rapidly than morphine and metabolism of both drugs occurs in the CNS.(McQuay H et al 1997; Chen ZR et al 1990; Oldendorf WH et al 1972)

4.4.4 Patient genotypes

It was not possible to show a link between the genotype/phenotype of the patients in the codeine group and the analgesic efficacy. One possible reason for this is the small number of patients recruited for this trial did not allow for appropriate statistical comparison. Of the 96 patients studied only four were PMs, with two in each group. This number of PMs (4.2%) was less than would be expected for a random UK population.(Ctytochrome P450, subfamily IID; CYP2D 1999) This may be explained by

the fact that we were using an inner city population with included a wide range of ethnic groups and the previously estimated figures for the UK are based mainly on Caucasian populations. This low number of PMs may also have occurred by chance, a problem that has been encountered by previous authors when having to select their populations at random and using small numbers of patients.(Morike K et al 1998; Chen S et al 1997)

From the results it can be seen in the codeine group that some patients who were EMs had little analgesic efficacy whilst some patients who were PMs or intermediate/poor metabolisers had good analgesic efficacy. This may suggest that enzyme activity is not the sole determinant of efficacy. Pain is a subjective experience and for some patients the use of NSAIDs may have been adequate analgesia on their own whilst for other patients the combination of opioid and NSAID was inadequate to provide analgesia. Also, from the results, it cannot be ruled out that codeine itself or one of its metabolites may be having an analgesic effect. These results may also highlight the difficulties associated with pain scoring and assessment of analgesic efficacy discussed earlier.

It was also not possible to demonstrate a link between genotype and plasma morphine and metabolite levels. Morphine was unable to be detected in patients of all phenotypes, not only the PMs. This could possibly be explained by the fact that the levels of morphine and its metabolites were low following codeine and were consistently around the limits of detection for the assay used. Thus in some of the patients in whom the results were ND, morphine and its metabolites may have been measured using a more sensitive assay.

Overall it is very difficult to draw any firm conclusions as to the effect of genotype on the analgesic efficacy of codeine from these results. Further studies involving more patients are required to allow for relevant statistical comparison.

Notes on recruitment

Ninety-eight patients were recruited into this study. Of which data was collected from 96, who were divided equally with 48 in each drug group. This figure was a long way

short of the intended 200 patients for this study. The reasons for this short fall were related to problems with recruitment:

- *A ban on elective adenotonsillectomy.* Unfortunately the trial had to be stopped earlier than expected. This was due to the ban placed on elective tonsillar surgery by The Department of Health and The Royal College of Otolaryngology in response to fears of transmission of variant Creutzfeld-Jakob disease (CJD) by the use of non-disposable equipment. At that time the Royal National Throat, Nose and Ear Hospital did not use disposable equipment and so the hospital decided to stop elective surgery until the disposable equipment became available. To date elective surgery has not recommenced.
- *Language problems.* As can be seen from table 16, the study population was multi-cultural as befits an inner-city London hospital. Consequently a proportion of patients and their families attending for surgery could speak little or no English. If the command of English was not adequate to allow informed consent or to understand the process of the trial and communication of pain scores then the patient was not recruited into the trial.
- *Non-attendance.* On average, for each surgical list, five patients were booked for surgery, though it was unusual for all five to attend. Often no reason was given or it was due to illness or social problems.
- *Refusal of consent.* Although this was not common some parents did refuse to give permission for their children to take part in the trial.
- *Exclusion criteria.* A number of patients were not recruited due to the fact that they fulfilled one or more of the exclusion criteria. Usually this was because they were outside the age range but pre-existing illness, such as obstructive sleep apnoea, and unsuitability for NSAIDs were also common.

4.4.5 Summary

Codeine and morphine appear to provide equivalent analgesia for children undergoing adenotonsillectomy at the doses prescribed with a decreased incidence of postoperative vomiting in the patients receiving codeine. However, on an individual patient basis, in comparison with morphine, codeine shows more variability in its effect and there is a small group of patients in whom it appears to provide little analgesia.

This variability may be associated with the wide range of metabolizing capacities of codeine within a population. However, this was not shown in this study. A number of factors may have hindered this process including small patient numbers, concurrent use of NSAIDs, the subjective nature of pain scoring and the difficulties associated with pain scoring. Though it is possible that the explanation for the variable efficacy of codeine is not as simple as just to be dependent on the extent of its metabolism to morphine. Further studies with larger numbers of patients are required to try and elucidate this.

Once again it was not possible to relate plasma morphine levels with effect. This, along with other evidence presented, suggests that many other factors are involved in the efficacy of opioids. However, further work looking at the levels of morphine and its metabolites over time following codeine administration in children may be warranted to allow us to understand more about the pharmacokinetics of the drug in this age group.

Overall it appears that both codeine and morphine can be used to provide analgesia for adontonsillectomy in this age group. Morphine is associated with a significantly higher incidence of vomiting but has a more consistent effect and does not appear to increase the incidence of sedation or respiratory depression. Codeine can be used successfully in a large proportion of patients, especially as part of a multi-modal approach to analgesia, but its use must be tempered with the realization that for some patients the analgesia may not be adequate.

CHAPTER 5. WORK ARISING FROM THE PREVIOUS STUDIES

5.1 Background to further study

As discussed previously codeine is frequently recommended for postoperative analgesia in paediatric medicine, especially for neonates and infants and in situations where airway management and neurological assessment are critical, due to the perception that it delivers good analgesia with a decreased incidence of non-analgesic opioid effects.(Semple D et al 1999; Husband AD et al 1996; Stoneham MD et al 1995; Hatch DJ et al 1995; Lloyd-Thomas AR 1990)

Results from the laboratory studies in this thesis using a rat animal model would suggest that there is developmental regulation of the efficacy of codeine in the rat. Although great care must be taken when extrapolating the results from animal work into humans this is an area that warrants further investigation. Pain assessment in non-verbal children such as neonates and infants is notoriously difficult and the clinical signs used to assess pain are often seen in and are easy to confuse with other situations such as hunger, cold, demanding attention etc. Thus in these age groups it is vital that we can be sure of the efficacy of the analgesic therapies we employ for pain relief.

Maturation of enzyme systems may be an important factor affecting the efficacy of codeine. The influence of development in humans on the efficacy and side-effects of codeine has not been well investigated. As discussed in chapter 1 it has been suggested that infants and neonates have a reduced metabolic capacity for codeine and that the activity of the CYP2D6 enzyme which carries out the O-demethylation reaction appears to be much lower in neonates than in adults.(Quiding H et al 1992; Jacqz-Aigrain E et al 1992; Ladona MG et al 1991; Treluyer J-M et al 1991; Ladona MG et al 1989)The glucuronidation pathway is also immature at birth and it has been shown that it continues to develop after the neonatal period.(McRorie TI et al 1992) How much activity of the CYP2D6 enzyme is required for analgesic efficacy is unknown though the work done in the rats suggests that when there is low activity of the enzyme converting codeine to morphine there is limited analgesic effect from a dose of codeine.

The aim of this study is to investigate the efficacy of codeine in neonates and infants when being used for postoperative analgesia and to look at their ability to convert codeine to morphine.

The operative model chosen for this study will be pyloromyotomy, which is a relatively commonly performed surgical procedure for neonates and infants with pyloric stenosis. Perioperative analgesia for this procedure is usually given in the form of simple analgesics such as paracetamol and the infiltration of local anaesthetic into the surgical wound with or without the use of opioid. The opioid of choice, if given, has traditionally been codeine due to its perceived advantages in this age group, as discussed above. If there is developmental regulation in the efficacy of codeine in humans then the use of this opioid may not add significantly to the analgesia received by the child and carry a potential risk of deleterious effects for no added benefit.

Aims

1. To compare the analgesic efficacy of two analgesic regimens in the early postoperative period for children undergoing pyloromyotomy, one with and one without codeine.

2. To asses the ability of children in this age group to metabolise codeine to morphine.

Hypothesis

1. There will be no difference between the two groups in terms of analgesic efficacy.

2. The metabolism of codeine to morphine will be reduced in this age group compared with older age groups.

5.2 Methods

This study will be based at Birmingham Children's Hospital and Great Ormond Street Hospital for Children. Approval is currently being sought from the respective local ethical committees and parental consent will be sought prior to enrolment of each child.

Recruitment will encompass children who are ASA 1 and 2 up to the age of three months who are undergoing pyloromyotomy. Either anticholinergic or no premedication will be given. Anaesthesia will be induced, at the discretion of the anaesthetist involved with the procedure, with either a gaseous or intravenous technique and once established endotracheal intubation will be performed following the use of muscle relaxation. A 22G cannula will be inserted either prior to or after the induction of anaesthesia to allow for blood sampling and intravenous access for the procedure. Maintenance of anaesthesia will be with an oxygen, nitrous oxide and isoflurane mixture plus bolus doses of muscle relaxant. At the end of the procedure muscle relaxation will be reversed using neostigmine 50mcg/kg and atropine 25mcg/kg. The patient will then be extubated awake breathing 100% oxygen.

The children will be randomised into two groups depending on which analgesic regimen they are to receive:

1. Group A: paracetamol 20 mg/kg at induction plus local infiltration of bupivicaine 0.25% into the surgical incision at the end of the procedure.
2. Group C: as above plus codeine 1mg/kg IM post induction of anaesthesia.

Other analgesia will only be given peroperatively if clinically indicated as assessed by the anaesthetist in charge of the case.

One hour after the injection 1-2 ml of blood will be withdrawn from the indwelling cannula, The blood will then centrifuged at 3000 rpm for 10 minutes within one hour of being taken. The plasma will then be pippeted off and stored prior to processing at -70°C. This plasma will be used for the measurement of plasma morphine levels by High Performance Liquid Chromatography. This is to be carried out at the Institute of Child Health by Innes Kastner and Dr Jonathan De-Lima, as described in Section 3.2.4.

The patient will be kept in the recovery area until they are able to return to the ward. Pain scoring will be performed in the postoperative period, either in the recovery room or on the ward by the nurse responsible for the care of the patient. Codeine phosphate or paracetamol will be charted for postoperative analgesia as per hospital protocol and will be given as required, as assessed by the anaesthetist or the nurse responsible for the care of the child.

Exclusion Criteria

Patients will not included in the study if they are:

1. ASA 3 or above
2. Using concurrent medication that may affect codeine metabolism
3. Currently being treated with other analgesic medication
4. Known to have a reason why the use of paracetamol and/or bupivacaine is contraindicated
5. Known to have had an adverse reaction to any of the drugs to be used

Outcome Measures

1. Pain scores - using the FLACC behavioural pain score.(Merkel SI et al 1997) (figure 19). The children in this trial will be non-verbal and thus a behavioural pain score is required that is appropriate for the ages being studied. The FLACC score is considered suitable for this age group and has been verified down to the age of two months.(Merkel SI et al 1997) It is also being implemented at both centres as the standard form of clinical pain scoring on the wards by the acute pain teams and nursing staff.

2. Time to first analgesia

3. Plasma levels of morphine and its metabolites

Analysis

Randomisation

Enrolled patients were randomised into either group A or group C using a standard simple randomisation to ensure equal numbers in both groups e.g. random permuted blocks using random numbers tables.(Altman DG 1991)

Power Calculation

From the FLACC score a value between 3 and 4 has been taken as the point which is "on the cusp" between patients in pain and those not in pain. Thus for the purposes of a power calculation the value of this difference, i.e. 1, will be taken as d. A standard deviation of 0.5 is being taken for pain scoring 60 minutes post analgesia(Merkel SI et al 1997). Thus for a significance level of 0.001 and a power of 95% the power calculation would be:

$$n = 2x17.81(0.5/1)^2$$
$$= 8.9$$

Thus the aim is to recruit 10 patients in each group.

Statistical tests

For continuous variables a t-test or ANOVA will be used, after a suitable transformation to remove skewnesss. The pain scores will be defined in terms of a single cut-off and the proportions above (or below) will be compared using chi-squared. Time to first analgesia will be analysed using survival analysis where analgesia equates to "death".

Categories	0	1	2
Face	No particular expression or smile	Occasional grimace or frown, withdrawn, disinterested	Frequent to constant quivering chin, clenched jaw
Legs	Normal position or relaxed	Uneasy, restless, tense	Kicking or legs drawn up
Activity	Lying quietly, normal position, moves easily	Squirming, shifting back and forth, tense	Arched, rigid or jerking
Cry	No cry (awake or asleep)	Moans or whimpers; occasional complaint	Crying steadily, screams or sobs, frequent complaints
Consolability	Content, relaxed	Reassured by occasional touching, hugging or being talked to, distractable	Difficult to console or comfort

When the child is awake, each of the five categories is scored from 0-2. This results in a total score between 0 and 10.

Table 19. The FLACC score(Merkel SI et al 1997)

Overall Conclusions

The evidence presented in the currently available literature suggests that the analgesic efficacy of codeine is wholly or mainly dependent on the metabolism of codeine to morphine and that codeine should probably be thought of as a prodrug for morphine. This metabolic conversion is catalyzed by the cytochrome P450 enzyme CYP2D6, the function of which has been suggested to be affected by both genetic variation and development. The aim of this thesis was to investigate the influence of these two factors on the analgesic efficacy of codeine using laboratory and clinical experiments.

P3 SD rats have been shown to have low activity of the CYP2D1 enzyme, which is thought to be the equivalent of the CYP2D6 enzyme in rats, whilst P10, P21 and P63-70 have much higher levels of activity. During experiments using various sensory and nociceptive stimuli P3 animals showed no response to codeine whereas the other age groups demonstrated a significant response, as did animals of all ages following morphine.

Thus overall the results show there is developmental regulation of the efficacy of codeine in rats and that an opioid effect is responsible for codeine efficacy. Though there are many possible reasons for differences in opioid effect during development, the lack of codeine efficacy in the P3 animals may suggest a lack of production of morphine from codeine at this age due to low activity of the enzyme converting codeine to morphine. However, we were unable to demonstrate decreased morphine production at this age due to technical difficulties with blood sampling in these smaller animals.

The clinical study compared the efficacy of codeine and morphine for analgesia following adenotonsillectomy in children. The results show that overall morphine and codeine appear to provide equivalent analgesia at the doses prescribed with a decreased incidence of postoperative vomiting in the patients receiving codeine. However, on an individual patient basis, in comparison with morphine, codeine shows more variability in its effect and there is a small group of patients in whom it appears to provide little analgesia.

However, it was not possible to link this variation with genetic variations in metabolizing capabilities. The results of this study may have been affected by a number of factors e.g.

small patient numbers, concurrent use of NSAIDs, the subjective nature of pain scoring and the difficulties associated with pain scoring. Though it is possible that the explanation for the variable efficacy of codeine is not as simple as just to be dependent on the extent of its metabolism to morphine.

Overall codeine can be used successfully in a large proportion of patients, especially as part of a multi-modal approach to analgesia, but its use must be tempered with the realization that for some patients, especially neonates and infants, the analgesia may not be adequate. Further work is required to further elucidate the role that genetic variation and development play in influencing the efficacy of codeine.

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